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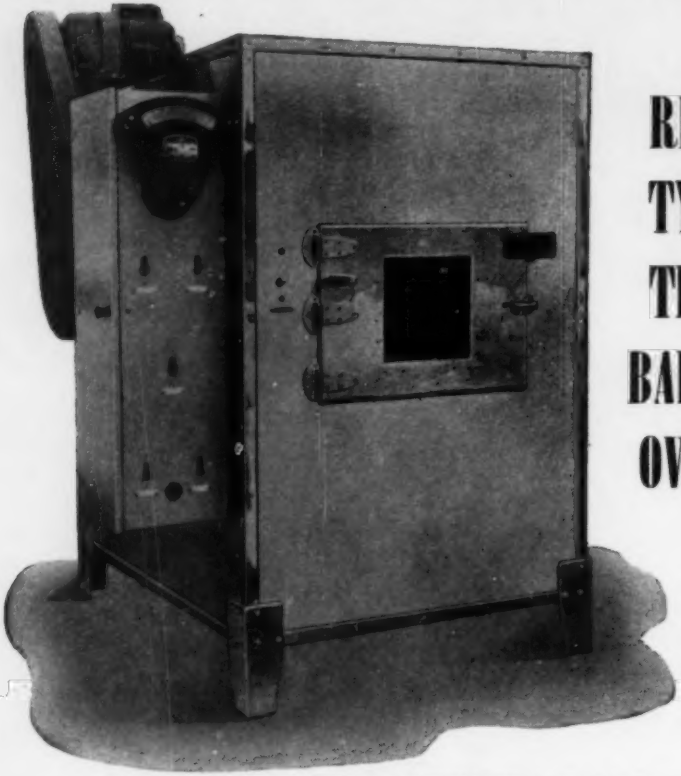

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CEREAL CHEMISTRY

VOL. 36 MARCH, 1959 NO. 2

PRESERVATION OF ROUGH RICE BY COLD STORAGE[1]

D. F. HOUSTON, R. E. FERREL, I. R. HUNTER, AND E. B. KESTER

ABSTRACT

Caloro rough rice with average moistures of 12.0, 13.1, 14.1, and 15.9% was stored in friction-top cans for 3 years at 34°, 20°, and −20°F. (1°, −7°, and −29°C.). Periodic determinations were made of moisture content, yeast and mold populations, sugars, acidity of the oil, and viability. Initial and final values were obtained for grade and milling yield. Yeast counts fluctuated markedly, and viability of the higher-moisture rices at 34°F. (1°C.) decreased during storage. Other characteristics varied slightly or inappreciably. The data offer evidence that experimental reference material can be suitably held for 2 or 3 years under cold storage, but suggest that for retention of viability over longer periods the rice should have less than 13% moisture when stored at temperatures as low as 34°F. (1°C.).

Cold storage of rough rice has potential value for at least two purposes: the prolonged maintenance of viability in breeding stocks of seed, and the retention of quality in reference material during extended research investigations. It is unlikely that refrigeration would be economical for commercial storages.

Extensive research on seed preservation and germination has shown that, in general, reduction in storage temperature and in seed moisture results in prolonged viability (2,8,10,13). Kondo and co-workers found that rough rice stored at low temperatures for 11 and 15 years retained many quality characteristics but failed to germinate (6,7). No information has been found, however, on the progressive results of rough rice storage at low temperatures. The cold storage of relatively moist rice here reported presents evidence on this point.

The question more frequently of concern to the research worker is whether composition and quality characteristics may be preserved for extended periods in reference samples of rice. He is also interested in knowing what storage conditions are necessary to maintain the material without appreciable change. This paper reports results bearing on these problems.

[1] Manuscript received April 8, 1958. Contribution from the Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Albany 10, Calif.

Materials and Methods

Caloro rice of the 1954 California harvest was taken directly from different lots at a single dryer with 11.9, 12.8, 14.5, and 15.6% moisture and showed slight variations in original characteristics. It was stored in friction-top cans at −20°, +20°, and +34°F. (−29°, −7° and +1°C.) within 2 days after sampling. The rice was slightly sour and of reduced viability because of a cool, wet harvest season.

Three subsamples were taken at periodic intervals from each storage sample of rice. A first portion of about 50 g. was taken with a sterile bottle from just below the surface of the rice for mold and yeast counts. A second 50-g. sample was taken for moisture determinations, and a third sample of about 150 g. was taken for chemical analysis. This sample was freed from extraneous material so that analytical data would be on the consistent basis of clean rough rice.

Counts of molds and yeasts were made with Difco malt agar by a previously described procedure (4). Moisture was determined on material ground to pass 20-mesh by drying a 10-g. sample at 104° to 106°C. for 16 hours in a forced-draft oven (5). Samples with over 13% moisture were dried below this level before grinding (two-stage procedure). Total and nonreducing sugars were determined as glucose by AOAC methods (1): A modified Ames and Licata titration (5) was used to determine acidity in the oil extracted from the rice with hexane; and viability was found by a modified paper-towel method of the U. S. Production and Marketing Administration (4). Milling yields and grade determinations on initial and final samples were made by official USDA procedures (12). No statistical treatments were attempted.

Results and Discussion

Initial, final, and some intermediate results are assembled in the accompanying table (Table I).

Moisture. Moisture contents remained constant within a few tenths of a percent. Average values were 12.0, 13.1, 14.1, and 15.8% at 34°F. (1°C.); 11.9, 13.1, 14.1, and 15.9% at 20°F. (−7°C.); and 12.0, 13.3, 14.1, and 15.9% at −20°F. (−29°C.). These average moisture contents are used to designate the several lots of rice in the tabulated results to provide ready intercomparison.

Grade and Milling Yields. The rice originally graded U. S. No. 1 except for the sour odor. There was no grade loss during storage, and the odors diminished. The driest rices were nearly free from sourness at the end of the test.

Yields of total and head rice were maintained or increased except

TABLE I

CHARACTERISTICS OF ROUGH RICE IN COLD STORAGE

	Storage Time	+34°F. Percent Average Moisture				+20°F. Percent Average Moisture				−20°F. Percent Average Moisture			
		12.0	13.1	14.1	15.8	11.9	13.1	14.1	15.9	12.0	13.3	14.1	15.9
	months												
Total milled rice (% of paddy)	0	74.3	74.1	72.4	68.5	74.3	74.1	72.4	68.5	74.3	74.1	72.4	68.5
	36	75.6	74.1	75.1	72.5	75.2	74.0	75.0	72.4	75.1	73.6	72.5	72.6
Head rice (% of paddy)	0	68.9	66.1	63.5	58.9	68.9	66.1	63.5	58.9	68.9	66.1	63.5	58.9
	36	71.0	66.1	68.2	63.5	70.1	66.4	67.6	62.1	69.5	64.4	66.6	62.0
Molds (thousands per g.)	0	<0.1	0.1	0.7	1.0	<0.1	0.1	0.7	1.0	<0.1	0.1	0.7	1.0
	36	0.4	0.1	5.0	<0.1	0.1	<0.1	18.0	0.2	0.3	0.2	0.1	<0.1
Reducing sugars (% as glucose, dry basis)	0	0.15	0.22	0.21	0.20	0.15	0.22	0.21	0.20	0.15	0.22	0.21	0.20
	12	.18	.23	.20	.22	.20	.24	.18	.20	.19	.24	.17	.21
	24	.15	.19	.16	.19	.15	.21	.19	.19	.15	.20	.19	.19
	36	0.10	0.18	0.14	0.09	0.07	0.08	.014	0.07	0.14	0.14	0.17	0.13
Nonreducing sugars (% as glucose, dry basis)	0	0.84	0.91	0.97	0.77	0.84	0.91	0.97	0.77	0.84	0.91	0.97	0.77
	12	.93	.97	.98	.86	.91	.96	.90	.88	.92	.98	.94	.90
	24	.89	.89	.91	.79	.89	.91	.92	.80	.89	.88	.92	.87
	36	0.88	0.88	0.86	0.82	0.87	0.95	0.89	0.86	0.80	0.91	0.89	0.81
Free acidity of oil (meq. acid per g. oil)	0	0.085	0.085	0.085	0.098	0.085	0.085	0.085	0.098	0.085	0.085	0.085	0.098
	12	.086	.084	.083	.098	.080	.085	.077	.095	.076	.078	.075	.076
	24	0.073	0.076	0.079	0.099	.069	.069	.071	.086	.063	.066	.061	.072
	36	0.080	0.079	0.078	0.115	0.074	0.073	0.075	0.090	0.070	0.071	0.068	0.086
Viability (% germination)	0	81	57	66	58	81	57	66	58	81	57	66	58
	12	81	61	73	69	79	64	70	63	80	66	75	69
	24	86	74	70	65	92	72	81	71	87	69	75	75
	36	83	46	58	33	87	62	76	49	83	52	71	73

for the sample at −20°F. (−29°C.), with 13.3% moisture. Here a slight, but probably insignificant, decrease occurred. Decreasing yields as sample moistures were greater resulted from milling the rice wet rather than after drying it to an optimum milling moisture. It is clearly evident from both total and head rice yields that a hardening occurred at all three temperatures during storage, which resulted in increased milling yields.

Molds and Yeasts. Initial yeast populations (1.1 to 13 millions per g.) were higher than those normally present on rice (3,9), which is an apparent reflection of the cool, wet harvest season. Mold counts were low, 1000 per g. or less. The record of the microfloral populations shows that molds underwent no consistent significant change, but did survive. Mold counts made with malt-salt agar have recently been reported (11) to be more satisfactory than those made with the presently used malt agar. Yeast counts during storage fluctuated markedly, and they varied between 7 thousand and 49 million per g. No useful conclusions can apparently be derived from these data. No attempt was made to determine species or strains of microflora.

Sugars. Sugar content, one of the most sensitive components of rough rice, showed almost no change.

Acidity. The sensitive characteristic of acidity in the oil showed slight but definite decreases during cold storage for all samples but the most moist at 34°F. (1°C.). Here a slight increase occurred, which shows that increases of acidity in wet rice can occur over extended periods, at least down to the freezing point of water. The decreases in acidity became greater as the storage temperature was lowered.

Viability. The initially rather low seed viabilities improved during storage under all conditions, and at the end of 3 years were higher than originally in seven instances. The increases tended to be larger as the temperatures were lower. They clearly indicate a post-harvest maturation, of which some evidence was reported previously (4) for higher storage temperatures.

Appreciable losses of viability eventually occurred for the three wettest samples at 34°F. (1°C.) and the highest moisture sample at 20°F. (−7°C.). A similar loss in the 13.3% sample at −20°F. (−29°C.) appears anomalous. The advantage of well-dried seed material for preservation of viability even at freezing temperatures is suggested.

Conclusions

The lack of important changes in sensitive characteristics of low-viability Caloro rough rice during 3 years of cold storage demonstrates that reference material with as high as 16% moisture content can suit-

ably be held for comparison in prolonged experimental programs. The minimum of change shown during storage at −20°F. (−29°C.) indicates that subfreezing temperatures would be most satisfactory for high-moisture rice.

For purposes of seed preservation, the information shows that viability can be retained in these relatively moist seeds for 2 to 3 years. The final viability and acidity values suggest, however, that for best preservation at temperatures as low as 34°F. (1°C.) the rough rice should be dried to somewhat below the 13% moisture level. This indication is in complete agreement with conclusions of previous workers (2,8,10,13), who have investigated the requirements for prolonged storage of viable seed.

Acknowledgments

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THE ESTIMATION OF STARCH PASTE FLUIDITIES[1]

W. R. FETZER[2] AND L. C. KIRST[2]

ABSTRACT

A new alkaline paste procedure is presented for estimating the degree of modification of both acid-modified and oxidized starches. The proposed method for Alkaline Paste Fluidity employs a new metal funnel with a stainless-steel orifice, increased concentration of alkali, standardized stirring for the pastes, and a procedure for obtaining the zero fluidity weight. The zero fluidity weight for the new funnel is 18.0 g. of starch. Two other weights of starch are used: 9.00 g. for unmodified corn starch and 54.0 g. for the highly acid-modified and highly oxidized corn starches. The three weights are multiples of 9. This has suggested a new method of nomenclature, composed of three digits, for identification of starches. The first digit is the multiplier of 9 used in the determination, and the other two digits are the fluidity; i.e., 265. It is believed that this method of assignment will simplify the reporting and understanding of fluidity data. A table of moisture-fluidity corrections for the range of 8 to 15% moisture is presented which enables the fluidity test to be started without waiting for the moisture result. Also included is a table of relative weights of modified corn starches in terms of common or unmodified corn starch which give approximately the same viscosity. These data are not absolute, since the properties of an aqueous paste are subject to many variables, but they are sufficiently reliable to permit many tentative formulations in commercial use. A number of other starch varieties have been evaluated by the new alkaline paste procedure.

No subject in starch chemistry has received more attention than the estimation of the fluid properties of starch pastes. Starch pastes usually form pseudo-plastic systems; nevertheless, their consistencies are referred to in terms of viscosity, or the reciprocal of viscosity—fluidity.

Prior to 1912, it was customary for starch manufacturers and their customers to evaluate the extent of acid modification (thinning) of a starch by cooking the starch in water to form a paste and thereafter determining the increase in fluidity by some physical means, usually the volume which would pass an orifice within a set time interval. There was no published method, and each investigator was left to devise his own methods and apparatus.

Buel, in 1912, discussed some of the properties of starch pastes (1). Although most starch chemists believe that the purpose of Buel's paper was to describe his funnel, this was not the primary reason for the publication. Buel showed that although the aqueous paste fluidity value was primarily a function of the starch, it was dependent upon other variables such as the rate of heating, duration of heating,

[1]Manuscript received April 17, 1958. This paper was presented at the 43rd AACC annual meeting, Cincinnati, Ohio, April 1958.

[2] Clinton Corn Processing Co., Clinton, Iowa, a division of Standard Brands, Inc.

amount of agitation, rate of cooling, and length of the cooling time. These manipulative variables often produced large differences in fluidity values which rendered the degree of modification of the starch uncertain. In its place he proposed a new method of paste procedure wherein a solution of sodium hydroxide was used to disperse the starch. He named his new method Alkaline Paste Fluidity.

The new procedure was comparatively simple, and it was widely accepted. Only a limited number of acid-modified or "thin-boiling" starches were in commercial use in 1912. Later, when commercial hypochlorite-oxidized starches were introduced, it was discovered that, although the alkaline paste fluidity was in good agreement with the aqueous paste fluidity for the acid-modified starches, the relationship did not hold for oxidized starches. This discrepancy contributed to the discrediting of the Buel technique, although most laboratories still evaluate acid-modified starches by the alkaline paste procedure. Insofar as the authors know, no data have been reported on the evaluation of oxidized starches by the alkaline paste method, nor have attempts been made to ascertain the reason for its failure.

Since then many publications (2) have appeared describing other methods for the estimation of starch fluidities and paste characteristics. Only a few of these procedures have survived. The funnel technique was neglected, although Morgan (4) published data procured through the use of the Buel funnel on the flow characteristics of an extended range of starch modifications, and Fetzer (2) reviewed the methods for evaluation of starch pastes. The continued use of the Buel funnel for 50 years, despite numerous innovations, indicates some very fundamental reasons for its survival.

The purpose of this paper is twofold: 1) to report the results obtained from an investigation of the basis for the alkaline dispersion of starch and 2) to present a new, simple, and reliable alkaline paste procedure for determining the extent of modification of both acid-modified and oxidized corn starch. Although alkaline dispersions of starch may be evaluated in any fluidity funnel, these investigations have been restricted to the Clinton funnel, which is regarded as a much improved form of the Buel funnel.

Aqueous Paste Fluidities

The aqueous paste fluidity may be determined cold or hot. Both methods require that the paste be cooked at a definite rate and for a fixed time, with controlled agitation during this period. The cold paste procedure requires that, after cooking, the paste be cooled to some definite temperature, e.g., 24°C. (75°F.), before the rate of flow

of the paste through the orifice is measured. This procedure requires further time and stirring. If the fluidity is determined hot, the paste is passed through the orifice usually at about 99°C. However, since viscosity differences between starch pastes diminish as the temperature increases, the temperature control must be precise in order to obtain reliable differences in viscosity. The cold paste evaluates the fluidity at lower temperatures; this increases the viscosity and makes differences in fluidity greater and thus easier to evaluate; but the cooling procedure involves stirring and possible "setback" or gel formation.

Hot Aqueous Paste Fluidity. The best example of hot aqueous paste fluidity is that obtained by the Hot Scott Viscometer, fully described by Kerr (3). The description covers the improved apparatus, which was designed to eliminate the many faults of the older equipment. The procedure for the Hot Scott calls for a very large number of sample weights of starch in order that the Hot Scott value may fall within the desirable range of the cup. There is no satisfactory way to correlate the various weights of starch with their respective values in terms of each other.

Cold Aqueous Paste Fluidity. The Clinton procedure for cold aqueous paste was used for all data reported in this paper for aqueous paste fluidities. The method is as follows:

Various amounts of starch are used; e.g., for common or unmodified corn starch, 9.50 g.; for modified starches, depending upon the extent of modification, 18.0, 35.0, and 50.0 g. One of these amounts is transferred to a 600-ml. Monel beaker. The starch is slurried in 307 ml. of tapwater,[3] and the beaker is placed in the cooker. Steam is applied at 3 lb. pressure with manual stirring until the paste "boils" (see second paragraph below). The pressure is reduced to maintain a rolling "boil" for exactly 1 minute, after which the beaker is removed to a cooling bath. The paste at 75°F. (24°C.) is transferred to the funnel. A small amount of paste is released through the orifice to ensure that no air bubbles are lodged in the stem. This paste is returned to the funnel. The paste level is then adjusted to the mark on the bowl indicating 300 ml., by release of the necessary amount of paste through the orifice. The flow of paste is started to a graduated cylinder. The amount obtained (in ml.) in 60 seconds, divided by 2, is the fluidity.

The cooking assembly (Fig. 1) consists of a steam chest and a special Monel beaker. The former is a heavily insulated vessel with an opening in the top. A thick rubber gasket is placed around the rim

[3] Tapwater (approximately 16 grains hardness) has always been used, on the assumption that this water simulates to some extent other municipal water supplies.

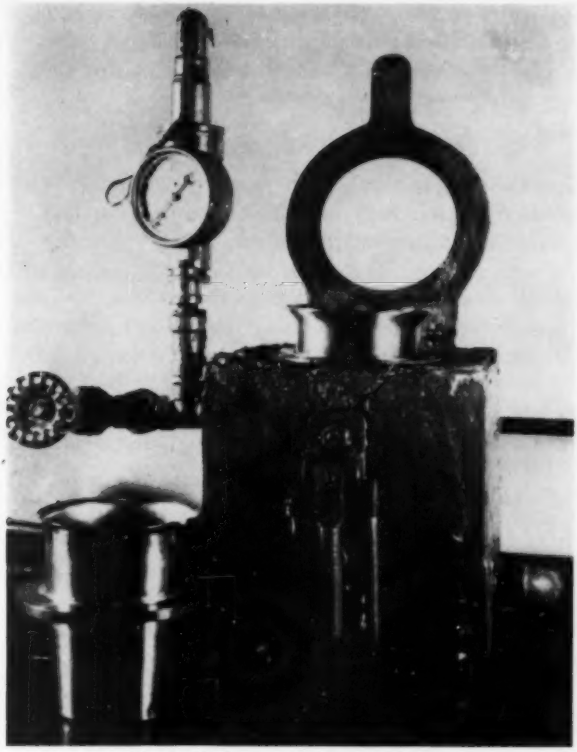

Fig. 1. Clinton aqueous paste cooker.

of the opening. The beaker is encircled by a bronze collar, about ½ in. wide and ¼ in. thick, firmly soldered in place. In operation, the beaker is centered in the opening. A tight seal between the beaker and the chest is obtained by means of a quick-opening clamp.

This cooking procedure was developed to eliminate manual stirring and to standardize the rate of heating and the uniformity of the "cook." However, because many modified starches cause excessive foaming, which appears to be "boiling," the maximum temperature is about 205°F. (96.1°C.), which necessitates the use of a thermometer to ensure that the same temperature is attained in all tests.

In addition to the variables mentioned by Buel, it is now known that ash or mineral matter and pH affect the aqueous paste fluidity. Thus, the authors return to Buel's original thesis; namely, that the aqueous paste fluidity carries too many variables in the paste prepara-

tion to make it a reliable and dependable method, and that the alkaline paste method eliminates most of these variables or reduces them to minor effects, so that only the modification of the starch is measured.

Buel's Alkaline Paste Procedure

Buel's alkaline paste procedure consists essentially of slurrying 2.50 g. of common starch or 5.00 g. of modified starch in 10 ml. of distilled water and adding thereto 90 ml. of 1.00% solution of sodium hydroxide with stirring. The water and the alkaline solution are at 25°C. and the mixture is held in a bath at 25°C. for 30 minutes. The paste is transferred to the funnel and the fluidity determined as the volume of paste (ml.) passing the orifice in 70 seconds.

The Buel funnel is shown diagrammatically in Fig. 2, and the essential data on which he based his alkaline paste procedure are shown in Table I. For reasons not disclosed, Buel standardized on

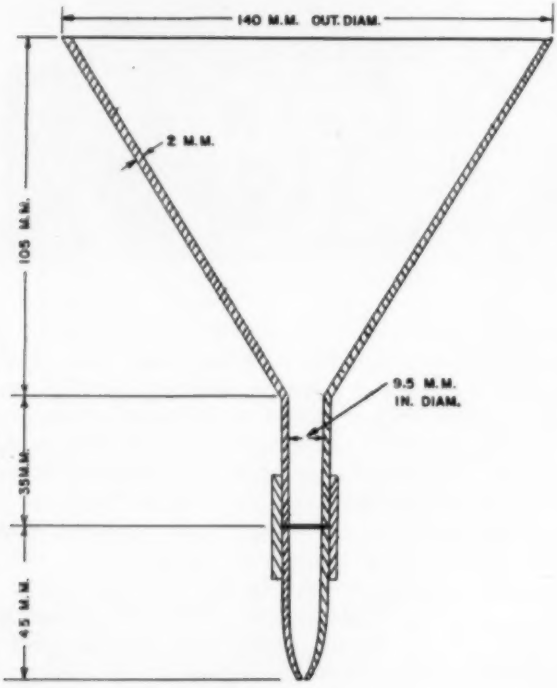

Fig. 2. Buel funnel.

TABLE I

ALKALINE PASTE FLUIDITY

(5.00 g. starch, 10 ml. distilled water, 90 ml. sodium hydroxide solution)

SODIUM HYDROXIDE	CONCENTRATION IN PASTE	FLUIDITY OBTAINED
g/100 ml	%	
0.50	0.45	No gelatinization
0.80	0.72	Partial gelatinization
0.90	0.81	34.1
1.00[a]	0.90	34.0
1.00[b]	0.90	37.9
2.00	1.80	53.5
3.00	2.70	54.2

[a] Buel; stirring at 75 r.p.m.
[b] Buel; stirring at 150 r.p.m.

1.00% sodium hydroxide solution. This practice has been followed to this time by most laboratories employing alkaline paste fluidities. Buel specified that the alkaline paste should be stirred at 70–80 r.p.m. for 3 minutes; the beaker is then transferred to a water bath, and after 30 minutes fluidity is determined. Most laboratories employ manual stirring, usually during the addition of the sodium hydroxide solution to the starch suspension and for some interval or intervals during the time the paste is in the water bath. The lack of a standardized stirring procedure is one of the causes for interlaboratory variance which have discredited the Buel technique.

Proposed New Funnel

One criticism of the Buel funnel is that the hydrostatic head on the orifice changes rapidly and not linearly as the paste level drops. This feature has restricted the range of useful numerical values to between 10 and 45, necessitating the use of different concentrations of starch to keep the paste fluidity within this range; there is little or no correlation between viscosity and the amounts of starch employed.

This criticism led Clinton, in 1928, to adopt a modified Buel funnel, and this design has undergone several modifications since that time. The original funnel was based on the combination of three separate units: a stem 160 mm. long to be placed between the bowl, which was the Scott cup, and the orifice tip of the Buel funnel. This design was an important improvement; it minimized the change in the hydrostatic head as paste passed the orifice. The method for standardization was changed from Buel's 110 ml. in 70 seconds to 200 ml. in 60 seconds. Since water must have a fluidity of 100, the relationship was obtained by dividing by 2 the water delivered and the volumes of pastes delivered in 60 seconds. This change was a further improve-

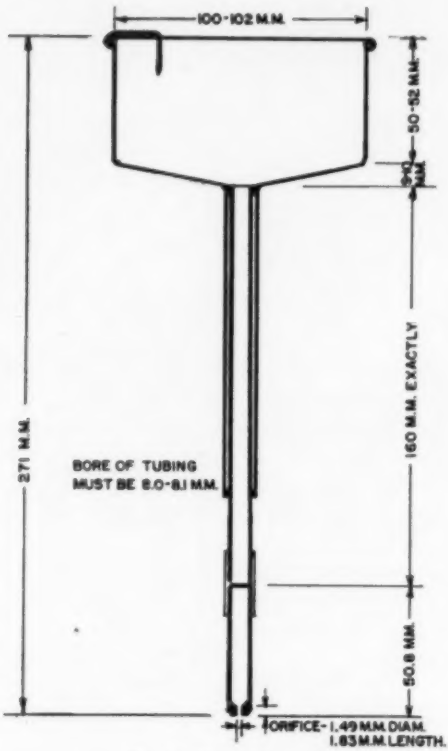

Fig. 3. Clinton funnel.

ment in funnel technique. However, the originators made a serious mistake. Buel's proportions of starch to water to caustic were employed, namely 15.0 g. of starch, 30 ml. of water, and 270 ml. of 1.00% sodium hydroxide solution.

The theory of the fluidity funnel requires that all modifications of starch fall in the range of zero to 100 fluidity. The weight of unmodified starch to be used for zero fluidity depends upon the design of the funnel. It is specific for that funnel only and must be determined experimentally.

Present Modified Buel Funnel. The funnel in use at Clinton at the present time, shown dimensionally in Fig. 3, has two forms of stem: in one it is precision-bore nickel tubing; in the other, precision-bore glass tubing.[4] The funnel, graduate, and beakers are shown in

[4] The reason for the two types of stem is that some analysts prefer the glass tube in order to see if there are entrapped air bubbles in the paste; other analysts wish to dispense with all glass.

Fig. 4. Assembly for alkaline paste fluidity.

Fig. 4. The stainless-steel orifice tip is machined from rod and has an i.d. of 8.00 mm. to the orifice. The orifice is bored by a No. 54 wire drill (1.40 mm.), which is slighty under the desired diameter.

Standardization of the Funnel. Approximately 300 ml. of distilled water at 24°C. are placed in the funnel. The level is lowered to that indicated by a pointer soldered to the inner surface of the funnel (1 cm. below the rim). Then the time required for the passage of 200 ml. to the graduated cylinder[5] is noted (usually over 60 seconds). The orifice is reamed and polished by rouge in successive tests until 200 ml. of water at 24°C. (100 fluidity) are passed in 60.0 ± 0.5 seconds. The exact time of the flow through the orifice becomes the standardiza-

[5] The graduated cylinder used is Corning No. 3065, distillate oil test D158. The capacity of this cylinder is 200 ml., graduated in 100 divisions. If this type of graduate is not available, an ordinary 200-ml. graduate may be used. The volume of water or paste must be divided by 2 to obtain fluidity.

tion time for that tip. The tip may be used on other funnels of this design without restandardization.

Calibration of the Funnel. The fluidity values as determined by the funnel are indices of the viscosity of the paste. An approximate idea of the viscosity of these pastes, in terms of centipoises, can be obtained by measuring the fluidity of sucrose solutions and referring the values so obtained to tables of viscosity. Since it becomes difficult to prepare solutions of sucrose which have a fluidity of 30 or lower, this range of the funnel was calibrated with standard oil samples of known viscosity as supplied by the National Bureau of Standards. The data obtained from the calibration curves are shown in Table II.

TABLE II

APPROXIMATE VISCOSITIES FOR VARIOUS FLUIDITIES

FLUIDITY	VISCOSITY	FLUIDITY	VISCOSITY
	centipoises		*centipoises*
5.0	500	45.0	67
10.0	275	50.0	54
15.0	215	55.0	43
20.0	178	60.0	35
25.0	147	65.0	27
30.0	121	70.0	21
35.0	100	75.0	16
40.0	82	80.0	11
		85.0	7

Materials and Methods

Starches. The theory of a fluidity funnel for the measurement of the degree of modification of starch requires that the unmodified starch used for the standardization of the funnel (zero fluidity) be selected with considerable care. The material chosen was produced at Clinton when both corn and milling conditions were regarded as satisfactory. It has been compared with samples of unmodified corn starch produced by other wet-millers, and it is believed to be a representative sample.

The acid-modified corn starches used were typical commercial products covering the entire range of modification. The oxidized starches included the highly oxidized products, often known as "gums." All were manufactured with sodium hypochlorite, and the finishing pH of the slurry was above 8.

Moisture was determined on all samples, so that the weight of the starch could be adjusted to 10.0% moisture basis.

Aqueous Paste Fluidity. The preparation of aqueous pastes has been discussed previously.

Alkaline Paste Fluidity—Manual Stirring. An appropriate quantity of starch in a 600-ml. beaker[6] is suspended in 150 ml. of water. To the slurry are added 150 ml. of 1.80 or 3.60% solution of sodium hydroxide,[7] with care to avoid unnecessary introduction of air bubbles.

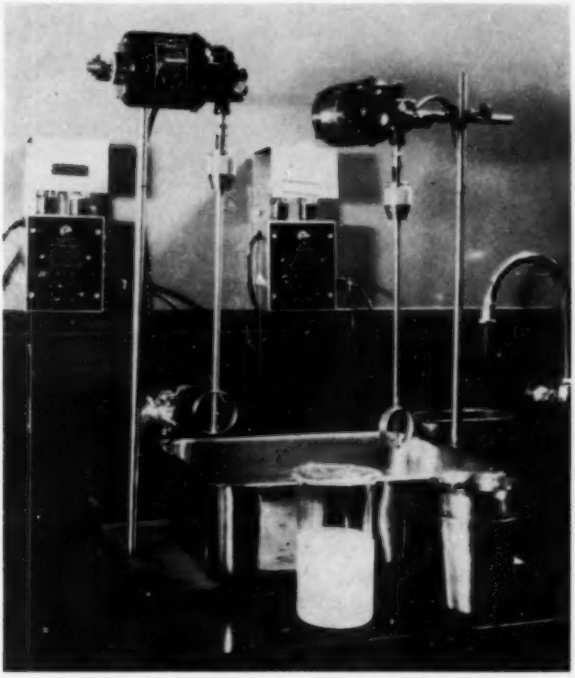

Fig. 5. Preferred assembly for stirring alkaline pastes.

The paste is stirred for 30 seconds and then placed in a water bath at 24°C. The paste is stirred for three 10-second intervals after 10, 20, and 30 minutes of elapsed time, respectively. Fluidity is determined after 30 minutes.

Alkaline Paste Fluidity—Mechanical Stirring. The solution of sodium hydroxide is added to the starch slurry and manually stirred for about 30 seconds, with the precautions noted above. Then the

[6] Either glass beakers, Corning No. 1100, or stainless-steel beakers, Arthur H. Thomas Co., No. 2103L, may be used.

[7] The first addition and concentration correspond to Buel's 90 ml. of 1.00% sodium hydroxide solution; the other to Buel's addition and concentration of 90 ml. of 2.00% sodium hydroxide.

beaker is placed in the water bath and the paste is mechanically stirred continuously for 15 to 30 minutes.

The choice of stirrer and propeller is important. The usual stirrer does not have sufficient torque to maintain a constant predetermined speed in heavy pastes. Several makes of motors and types of propellers were tried. The most satisfactory combination is shown in Fig. 5[8].

Results

Effect of Stirring. Buel's data on the effect of stirring covered a single sample of starch, and this sample was not identified as to type. This variable has been studied on a large selection of starches. Tables III and IV indicate that the effect of stirring is more pronounced on thick pastes, i.e., unmodified and the lower-oxidized starches. Stirring has the least effect on the acid-modified starches; in fact, it appears that there is little or no difference between manual and mechanical stirring.

Effect of Increased Alkalinity. Buel reported data on the effect of increased alkalinity, but again the data covered a single sample of an unidentified starch. This effect has been investigated for a large selection of starches (Table V). The results show that increased concentration of sodium hydroxide has its greatest effect on unmodified starch and the lower acid-modified and the lower-oxidized starches. Again, if the modification is high enough, an increase in alkalinity has little or no effect.

TABLE III

EFFECT OF STIRRING ON THE ALKALINE PASTE FLUIDITY OF COMMON CORN STARCH

(1.80% Sodium hydroxide, holding time 30 minutes)

STARCH	FLUIDITY VALUES				
	Manual Stirring	Mechanical Stirring (r.p.m.)			
		100	150	200	250
g/300 ml					
6.00	32.5	36.0	43.0	49.0	48.5
8.00	16.0	20.0	30.0	36.0	36.5
10.00	9.0	14.0	20.0	26.0	27.0
12.00	5.0	9.5	14.0	18.0	18.0
15.00	2.0	4.0	7.0	7.0	8.0

[8] The recommended stirrer is a DC motor, operated by two 2050 thyratron tubes, turning the shaft through an 18:1 gear reduction. The speed of the motor is determined by dial settings on the control box (G. K. Heller, Las Vegas, Nevada). The stirrer is equipped with a hoop-type propeller (Arthur H. Thomas Co., 9240 U). The dimensions and action of this propeller function extremely well with the 300 ml. of paste contained in a 600-ml. dye pot beaker.

Another type of motor that is also recommended is manufactured by the Bodine Electric Company of Chicago. It is a split-phase reducer motor with these specifications: Catalog No. B 2222X-8½H; type NSI-12 RH; 1/50 HP; 115 volts AC; motor, r.p.m. 1725; gear ratio 8½:1; r.p.m. no load 215, full load 205; torque 4.3 inch-pounds.

TABLE IV

EFFECT OF TIME OF STIRRING ON THE ALKALINE PASTE FLUIDITY OF COMMON CORN STARCH AND ITS MODIFICATIONS

(1.80% Sodium hydroxide, holding time as specified)

TYPE		STARCH	FLUIDITY VALUES			
			Manual Stirring, 30 Minutes	Mechanically Stirred (200 r.p.m.)		
				15 Minutes	30 Minutes	45 Minutes
		g/300 ml				
Common		9.00	10.5	29.5	35.0	36.0
		9.00	10.5	27.5	29.5	30.5
Acid-modified:	10	18.00	10.5	11.0	12.5	...
	30	18.00	32.0	33.0	34.0	...
	40	18.00	37.5	38.0	38.0	...
	60	18.00	59.5	59.3	59.3	...
	90	18.00	84.0	83.5	84.0	...
Oxidized:	10	18.00	6.25	8.0	11.5	...
	30	18.00	22.5	23.0	33.5	...
	50	18.00	48.0	44.5	51.0	...
	80	18.00	78.0	80.0	80.0	...
D "gum"		50.00	67.0	67.5	68.0	...

TABLE V

COMPARISON OF ALKALINE PASTE FLUIDITIES OBTAINED WITH 0.90 AND 1.80% SOLUTIONS OF SODIUM HYDROXIDE

DESIGNATION		STARCH	MANUALLY STIRRED HOLDING TIME 30 MINUTES		MECHANICALLY STIRRED, 200 R.P.M. HOLDING TIME 30 MINUTES	
			0.90%	1.80%	0.90%	1.80%
		g/300 ml				
Common		9.00	8.0	13.5	12.5	35.0
Acid-modified:	30	18.00	13.5	32.0	14.0	33.0
	60	18.00	44.0	60.0	48.0	59.3
	90	18.00	85.0	84.0	85.0	84.0
Oxidized:	30	18.00	13.0	22.5	16.0	33.5
	50	18.00	32.0	48.0	41.0	51.0
	80	18.00	75.9	77.0	77.0	80.0

TABLE VI

EFFECT OF TEMPERATURE ON THE ALKALINE PASTE FLUIDITIES OF DIFFERENT STARCHES

(1.80% Sodium hydroxide mechanically stirred at 200 r.p.m.; holding time, 30 minutes)

TYPE OF STARCH	STARCH	FLUIDITY OBTAINED AT:		
		70°F.(21.1°C.)	75°F.(24.0°C.)	80°F.(26.7°C.)
	g/300 ml			
Common corn	10.00	24.0	26.0	29.0
	8.00	35.0	37.0	40.0
	6.00	47.5	48.5	50.0
	4.00	61.0	62.5	63.5
Oxidized corn	50.00	66.5	68.0	69.0

Effect of Temperature. Temperature control for the alkaline paste procedure is simple: the starch is slurried, the alkali added, the paste retained, and the fluidity determined, all at the same temperature. However, the magnitude of the effect of temperature was studied; the data in Table VI indicate that the effect is small.

Zero Fluidity. The amount of unmodified corn starch in pasted form which will just pass the orifice may be established by extrapolating a plot of the fluidity obtained with increasing amounts of starch in the range of 100 to 10 or less, to zero fluidity. This was carried out by employing 2.00-g. increments of starch with three pasting procedures: A, alkaline paste (1.80%) manually stirred; B, alkaline paste

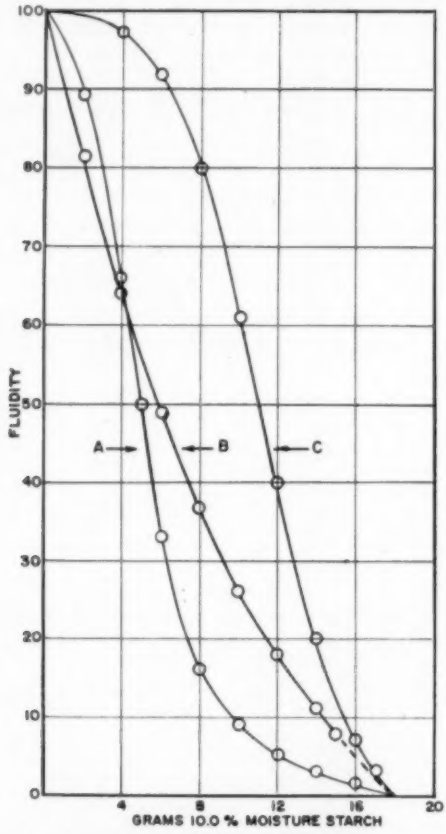

Fig. 6. Amount of starch to produce zero fluidity. A, alkaline paste — manual stirring; B, alkaline paste — mechanical stirring; C, aqueous paste.

TABLE VII

ALKALINE PASTE FLUIDITIES OF COMMERCIAL ACID-MODIFIED CORN STARCHES
(1.80% Sodium hydroxide mechanically stirred at 200 r.p.m.; holding time, 30 minutes)

	STARCH (grams per 300 ml.)								
	5.00	10.00	18.00	20.0	30.0	40.0	50.0	60.0	70.0
Common	56.0	26.0	0						
Acid-modified	74.0	37.5	11.0						
	85.0	62.0	32.0	26.0	0.0				
	87.5	73.0	45.0	38.0	13.0				
	91.0	78.0	55.0	50.0	27.0				
	92.0	82.0	62.0	57.0	34.0				
	93.0	85.0	71.0	68.0	48.0	31.0			
	94.5	87.0	78.0	75.0	61.0	48.0	34.0		
	96.0	92.0	84.0	82.0	72.0	62.0	52.0	42.0	
	97.5	97.0	87.0	85.5	77.0	69.0	61.0	52.0	

TABLE VIII

AQUEOUS PASTE FLUIDITIES OF COMMERCIAL ACID-MODIFIED CORN STARCHES

	STARCH (grams per 300 ml.)							
	5.00	10.00	18.00	20.0	30.0	40.0	50.0	60.0
Common	92	59.0	0.0					
Acid-modified	93.0	61.0	6.0	0.0				
	94.0	84.0	28.0	14.0				
	95.0	87.0	38.0	22.0	0.0			
	95.0	89.0	49.0	32.0	0.0			
	96.0	91.0	60.0	47.0	0.0			
	97.0	93.0	71.0	62.0	8.0	0.0		
	98.0	94.0	82.5	77.0	20.0	0.0		
	99.0	97.0	90.0	88.0	62.0	8.0	0.0	
	99+	98.0	92.0	90.0	72.0	38.0	3.5	0.0

(1.80%) mechanically stirred; and C, aqueous paste. The holding time in all cases was 30 minutes. Figure 6 indicates that the weight of unmodified starch to produce zero fluidity for this funnel is 18.0 g.

The curves also give considerable information about the conditions for a fluidity determination. The ideal curve for the relationship of fluidity vs. weight of starch would be a straight line drawn between 100 fluidity (water) and zero fluidity (18.0 g. of starch). Curve *B* (Fig. 6), obtained by alkaline paste dispersion, mechanically stirred, is close to the ideal straight line. The "flat" parts in curve *A* (15 to 0 fluidity; 8 to 18 g. starch) and in curve *C* (100 to 90 fluidity; 0 to 6 g. starch) are not favorable for a fluidity determination, for too much starch is required to produce a small change in the fluidity value. This situation is particularly unfortunate for the alkaline paste dispersion, manually stirred, for these are the conditions which determine whether a starch has been slightly modified, a situation which has plagued the

starch industry for years. The steep parts of curves *A* and *C* also indicate difficulty for a fluidity determination, for under these conditions the fluidity changes rapidly for a small change in the amount of starch used; i.e., reproducibility of the measurement is not easy to obtain.

Acid-Modified Corn Starches. A family of fluidity curves was obtained for a series of acid-modified corn starches by the alkaline paste procedure (mechanically stirred, 200 r.p.m.) and by the aqueous paste procedure. For the lowest acid-modified corn starch this was done by employing weights of starch in increasing 2.00-g. increments; for the higher-modified starch, 10-g. increments. The data so obtained were plotted, and the best curve was drawn through the points. From these curves of fluidities, tables have been prepared giving the fluidity values for various amounts of starch – alkaline paste fluidities, Table VII; aqueous paste fluidities, Table VIII.

Oxidized Corn Starches. Another family of fluidity curves was obtained for a series of oxidized corn starches as described above. The data appear in Tables IX and X.

TABLE IX

ALKALINE PASTE FLUIDITIES OF COMMERCIAL OXIDIZED CORN STARCHES

(1.80% Sodium hydroxide mechanically stirred at 200 r.p.m.; holding time, 30 minutes)

	STARCH (grams per 300 ml.)								
	5.00	10.00	18.00	20.0	30.0	40.0	50.0	60.0	70.0
Common	56.0	26.0	0.0						
Oxidized	62.0	37.0	11.5						
	73.0	57.0	33.5	29.0					
	79.0	66.0	48.0	45.0	25.5				
	95.0	90.0	80.0	78.0	65.0	51.0	35.0		
	96.0	92.0	84.0	82.0	72.0	62.0	52.0		
	97.0	94.0	87.0	85.5	77.5	69.0	61.0	53.0	41.0
	98.0	95.0	89.5	88.0	81.0	74.0	67.0	60.0	53.0

TABLE X

AQUEOUS PASTE FLUIDITIES OF COMMERCIAL OXIDIZED CORN STARCHES

	STARCH (grams per 300 ml.)								
	5.00	10.00	18.00	20.0	30.0	40.0	50.0	60.0	70.0
Common	92.0	59.0	0.0						
Oxidized	93.0	64.0	5.0						
	94.0	79.0	32.0						
	94.5	82.0	49.0	40.0					
	96.0	90.0	77.5	74.0	52.0	28.0	9.5		
	97.0	93.0	88.0	87.0	73.0	54.0	32.0	23.0	
	98.0	95.0	90.0	88.0	79.0	68.0	54.0	40.0	
	99.0	97.0	93.0	92.0	85.0	77.0	68.0	59.0	46.5

Evaluation of Modified Corn Starches by a New Alkaline Paste Procedure

The researches which have been described have led to a new procedure for alkaline paste fluidities, based on certain modifications of the Buel method; namely, an improved funnel, increased concentration of alkali, mechanical stirring, and the use of a "zero fluidity weight" of starch — 18.0 g. Two other weights have been assigned: 9.00 g. for common corn starch, 54.0 g. for highly acid-modified or oxidized starches. All of these weights are multiples of 9. Thus, if the analysis of three starches — common, modified, and highly modified — gave identical fluidities, e.g., 35, their respective fluidities would be reported as 135, 235, and 635. The new procedure enables a correlation of the three amounts of starch in terms of each other, as shown in Table XI.

TABLE XI

PROPOSED EVALUATION OF ACID-MODIFIED AND OXIDIZED CORN STARCHES BY ALKALINE PASTE FLUIDITY

DESIGNATION		ALKALINE PASTE FLUIDITY		
Acid-Modified	Oxidized	9.00 g.	18.00 g.	54.0 g.
130[a]	130[a]	26–34	0–5	0
210	210	(40–43)	10	
220	220		20	
230	230		30	
240	240		40	
250	250		50	
260	260		60	
270	270		70	
280	280		80	(30)
650	650 or A "gum"		..	50
660	660 or B "gum"		..	60
	665 or C "gum"		..	65
	670 or D "gum"		..	70

[a] Average value for unmodified corn starch.

Determination. Transfer 9.00, 18.00, or 54.0 g. of starch to a 600-ml. beaker. Add 150 ml. of distilled water and stir thoroughly to effect a uniform slurry. Add 150 ml. of a 3.60% solution of sodium hydroxide and continue manual stirring until the caustic is uniformly distributed, about 10–15 seconds. Transfer the beaker to a water bath maintained at 24°C. (75°F.) and mechanically stir for 30 minutes, employing a hoop propeller, at 200–225 r.p.m.

Transfer paste slowly to funnel to avoid introduction of air bubbles. Release a small quantity of paste through orifice to eliminate any air bubbles that may have been trapped in the stem. Return this paste to bowl. Withdraw paste through orifice until surface of paste in bowl is at level indicated by metal pointer. Place graduate beneath orifice at same distance employed in the standardization. Measure amount of paste passing orifice in standardization time (about 60 seconds).

Determine moisture of starch by one of the accepted procedures. If the moisture is other than 10.0%, correct fluidity to a 10.0% basis by the values which appear in Table XII.

TABLE XII

FLUIDITY CORRECTIONS FOR MOISTURES OTHER THAN 10.0 PERCENT

STARCH	OBSERVED FLUIDITY	PER CENT MOISTURE IN STARCH						
		8.0	9.0	11.0	12.0	13.0	14.0	15.0
g./100 ml.		Add		Subtract				
9.00	32	1.0	0.5	0.5	1.0	1.5	2.0	2.5
18.00	10	1.0	0.5	0.5	1.0	1.5	2.0	3.0
18.00	30	1.0	0.5	0.5	1.0	1.5	2.0	2.5
18.00	40	1.0	0.5	0.5	1.0	1.5	1.5	2.0
18.00	50	1.0	0.5	0.5	0.5	1.0	1.0	1.5
18.00	80	0.5	0.0	0.0	0.5	0.5	1.0	1.0
54.0	35	2.0	1.0	1.0	1.5	2.5	3.0	4.0
54.0	50	1.0	0.5	0.5	1.0	1.5	2.0	2.5
54.0	70	1.0	0.5	0.5	1.0	1.0	1.5	2.0

Discussion

Buel's choice of a paste alkalinity of 0.90% is puzzling, since his data indicated 1.80%. Still more puzzling is the failure of starch chemists to recognize this key to an improved alkaline paste procedure. For if the alkaline paste procedure is to have maximum utility, this increased concentration of alkali must be employed. The differentiation in fluidity of starches in the early stages of modification is greatly improved. Dispersion of the lower-oxidized starches is much better. These starches form thick pastes which are stringy and mucilaginous. Buel's concentration often produces lumpy pastes which give erratic fluidity data. The increased concentration of alkali produces an immediate and complete dispersion of the starch. The hard particles which are often present in commercial starch are only slowly dispersed in the lower concentration. Most important of all is the fact that the highly modified starches can be evaluated on an increased-weight basis. It is impossible to disperse amounts of starch as great as 54.0 g. in Buel's concentration of alkali.

The authors believe that the reliability of the alkaline paste dispersion on any funnel can be improved by increasing the concentration of the alkali to 1.80% and by mechanical stirring under standardized conditions.

Slurrying the starch in a larger amount of water than specified by Buel and compensating for the additional water with a stronger concentration of sodium hydroxide reduces the possibility of lumpy pastes and facilitates the procedure.

The amounts of starch used in the procedure may be changed to 10.00, 20.00, and 60.0 g., provided the amounts of both water and alkaline solution are increased to 167 ml. An orifice tip is regarded as satisfactorily polished when the time for the flow of 200 ml. of water

falls within the range of 60 ± 0.5 seconds and when, after about 135 ml. of water have been delivered, the stream quietly enters the surface of the water in the graduated cylinder.

Various types of propellers have been tried, varying from the simple paddle to the ribbon type. The hoop type was judged to be the best. One point is essential; namely, the propeller should not whip air into the paste, for there is some evidence that this may cause a measurable thinning in heavy pastes, which do not readily release air to the surface.

The data indicate that the stronger concentration of alkali and mechanical stirring will reduce the time for an alkaline paste fluidity in some instances to 15 minutes.

The quality of unmodified starch produced by any wet-miller is often dependent upon the corn received, which varies according to the growing season, maturity, harvesting, etc. Other starches cannot be expected to give the same relationship that has been established for corn starch. However, data are shown on other starches in the table below.

Alkaline Paste Fluidities of Various Unmodified Starches
(9.00 g. starch; 10.0% moisture)

Starch	*Alkaline paste*	*Starch*	*Alkaline paste*
Corn	26–34	Tapioca	47.0
Rice	25.5	Tapioca	44.0
Wheat	28.0	Potato	12.5
Wheat	51.0	Potato	15.5
Waxy maize	16.5	Sago	53.0
Waxy maize	10.5	Arrowroot	31.0

The samples are known to be authentic materials, but it cannot be stated with certainty that they have not been modified to some degree in processing.

The data reported in Tables VII and IX enable the calculation of alkaline paste fluidities to a 10.0% moisture basis when the moisture of the sample is other than 10.0% and within the range of 8.0 to 15.0%. The corrections for acid-modified starches below 250 are not exactly the same for their respective fluidities, but the differences are within the tolerance for the determination.

The highly oxidized starches reported here, that is, those which require 54.0 g. for their evaluation, are often called "gums." As a group they are unique, for the plot of alkaline paste fluidity vs. weight of starch in the range of 10 to 60 g. is a straight line. This is not the usual pattern for starches and suggests Newtonian flow.

An important use of fluidity data is to make available a table of the relative amounts of the various modified corn starches which will pro-

TABLE XIII

RELATIVE AMOUNTS OF MODIFIED CORN STARCHES (10% MOISTURE BASIS) REQUIRED TO PRODUCE PASTES OF 50 FLUIDITY (AT 24°C.)

FLUIDITY	ACID-MODIFIED CORN STARCH		OXIDIZED CORN STARCH	
	Percent by Weight	Ratio	Percent by Weight	Ratio
130[a]	3.4	1.00	3.4	1.00
210	3.85	1.15	3.85	1.15
220	4.3	1.25	4.3	1.25
230	4.8	1.40	4.8	1.40
240	5.2	1.55	5.2	1.55
250	5.8	1.70	6.0	1.75
260	6.4	1.90	6.7	2.00
270	7.2	2.10	7.7	2.25
280	8.1	2.40	9.5	2.80
650	9.4	2.75	11.6	3.4
660	10.8	3.20	14.1	4.15
665	...	...	17.0	5.0
670	...	...	20.4	6.0

[a] Common or unmodified corn starch.

duce commercial pastes of equal viscosity. Since the viscosities of aqueous pastes are subject to numerous variables in their preparation, such data are more to guide the user of starch than to provide absolute values for commercial pastes. To conform to the usual practices of starch formulation, these calculations have been made on percent by weight of 10% moisture starch (Table XIII).

Some starch chemists maintain that a starch must be evaluated by the aqueous paste fluidity, because the customer uses a water paste. However, the customer's methods of making a starch paste, the water used, and the treatment of the paste after it is made are so different from those employed in laboratory evaluation that it is difficult to believe that they bear a consistent relationship to laboratory values. The authors believe that the better policy would be to make a series of modified starches, in which each member increases in degree of modification; to evaluate each one by a method which measures *only* the degree of modification of the starch; and to select the starch needed for a particular method of paste preparation and application.

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FURTHER STUDIES ON THE DETERMINATION OF SULFHYDRYL GROUPS IN WHEAT FLOURS[1]

H. A. SOKOL, D. K. MECHAM, AND J. W. PENCE

ABSTRACT

Maximum and reproducible sulfhydryl values for flours were obtained by amperometric titration only when sample preparation conditions were selected to avoid losses of sulfhydryl prior to the actual titration as well as to make less accessible groups readily available. Requirements include (a) dispersion and titration of the sample at 2°C. instead of room temperature, (b) use of flour dispersions in 6 to 8*M* urea rather than extracts or digests, and (c) use of ethylenediaminetetraacetate to minimize the effect of interfering metallic ions. Under these conditions, titration with silver nitrate at pH 7.5 (Benesch method) showed the sulfhydryl content in flour dispersions to be at least twice that in buffer extracts or trypsin digests.

Sulfhydryl contents of the flour samples tested ranged from 0.66 to 1.30 microequivalents per g. of flour, or from 6.1 to 10.3 microequivalents per g. protein.

In a previous investigation (6), three methods for the determination of sulfhydryl groups were compared as to their suitability when small amounts must be measured in the presence of the various constituents of wheat flours. For that purpose, flours were digested with trypsin to solubilize the flour proteins. Although the enzyme digestion procedure appeared to be satisfactory with respect to the recovery of sulfhydryl groups from bovine serum albumin digested in the absence and presence of flour, later experience showed that this was not true with flour proteins. Consequently, the measurement of the total sulfhydryl content of flours and flour fractions has required further investigation.

In particular, the lability of some of the sulfhydryl groups in flour has necessitated examination of the conditions for preparation of sam-

[1] Manuscript received April 14, 1958. Presented at the 43rd annual meeting, Cincinnati, Ohio, April 1958. Contribution from Field Crops Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Albany 10, Calif.

ples and titrations to avoid rapid losses. Procedures giving maximum recovery of sulfhydryl groups have been established on the basis of observations described in this paper.

For the present studies, only the silver amperometric titration method (2) has been used. It was preferred to the mercuric chloride method (5) because the latter requires 1) complete absence of free oxygen during the titration, and 2) different conditions of pH and salt concentration for sulfhydryl compounds of low molecular weight from those used for high; whereas neither consideration applies to the silver-tris method.

Materials and Apparatus

Materials. Three unbleached flours were experimentally milled from Lee, Elgin, and Idaed wheat; these flours contained 15.1, 11.9, and 12.4% protein, respectively. Three unbleached commercially milled flours were used: bread (hard red spring), cookie, and cracker flours containing 12.7, 6.7, and 10.0% protein, respectively. A commercial mill also provided three flours from separate mill streams: fifth break, second middlings, and second low-grade flours.

In some of the specificity tests, prime starch made by the batter process at the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, was used.

Soluble and crude starch fractions were washed from Lee flour. These washings were centrifuged, separated, and dried. Gluten from Lee flour was lyophilized and then ground to pass a 40-mesh screen. Performic-acid oxidation of the solubles and gluten was carried out as described by Schram *et al.* (7).

Glutathione was from Eastman Organic Chemicals (Rochester 3, N.Y.)[2]; tris (hydroxymethyl) aminomethane from Sigma Chemical Co. (4648 Easton Ave., St. Louis 13, Mo.); and disodium ethylenediamine tetraacetate (EDTA) from Versenes, Inc., Framingham, Massachusetts.

Apparatus. The apparatus was, for the most part, the same as that of Benesch (2). However, a Leeds and Northrup Type E Electrochemograph was used. With this instrument, a permanent record of the entire titration sequence is obtained. A magnetic mixer was used for sample stirring. A Sargent hook-type electrode gave the greatest sensitivity, probably because of its large surface area and greater tolerance to fouling from any insoluble material in dispersions being titrated.

Method

Sufficient sample to provide 0.15–1.5 microequivalents of sulfhydryl

[2] Mention of trade names does not constitute endorsement by the Department of Agriculture over others of a similar nature not mentioned.

was combined with 3.4 ml. 1*M* nitric acid, 4.0 ml. 1*M* tris (hydroxymethyl) aminomethane, and 0.3 ml. 1*M* potassium chloride in a 100-ml. beaker placed in an ice bath. The sample was dissolved or dispersed in this mixture with a magnetic stirrer. Cold 8*M* urea solution, or solid urea and water, then was added to give a final volume of 35–40 ml. (urea concentration, 6.5–8.0*M*), followed by 1.0 ml. of 0.03*M* EDTA. The platinum electrode was immersed in the titration mixture and rotated at approximately 600 r.p.m. When the current stabilized at a constant value, 0.05- or 0.10-ml. increments of 0.001*M* silver nitrate were added at 1-minute intervals. The titration mixture was stirred with the magnetic mixer for 20 seconds after each addition, and the current value was recorded 40 seconds after discontinuance of mixing. The intercept of the excess reagent line with the base line (current in the absence of excess silver) was determined graphically to measure the volume of titrant consumed.

Application of Method

Titration of Flour Dispersions. The use of flour dispersions or suspensions had earlier been avoided in the belief that complete reaction of sulfhydryl groups and titrating agent would not be obtained unless flour proteins were completely in solution and remained so during titration. This does not appear to be a difficulty in the silver-tris titration, however, as shown by comparison of titrations made in the presence and absence of urea:

	Sulfhydryl Content in Microequiv. per g.	
	No urea	*Urea*
Elgin flour	0.73	0.74
Hard red spring flour	1.13	1.05

Urea was added originally to disperse and denature flour proteins and so to make available for titration a maximum number of sulfhydryl groups. Flour-buffer dispersions gave a sulfhydryl titer approximately equal to that of the flour-buffer-urea dispersions, although in the absence of urea the beginning of the excess reagent line was less clearly defined. Consequently, all sulfhydryl groups in flour proteins appear to be available to silver even when some of the proteins are not in solution, although less readily than after dispersion in urea.

Effects of Temperature and Chelating Agent. A rapid loss of sulfhydryl occurs in flour-buffer-urea dispersions when the dispersion is prepared at room temperature, as shown by the following comparison of values obtained at room temperature and 2°C.:

	Sulfhydryl Content in Microequiv. per g.	
	25°C.	*2°C.*
Second middlings	0.44	0.66
Fifth break	1.09	1.30
	Equivalents per mole	
Glutathione	0.93	1.00

Results at 2° and −5°C. were identical, but the former temperature was preferred for subsequent use because of its convenience.

This marked effect of temperature was first suspected from decreasing values for serial titrations of duplicate samples as they became warm from the magnetic stirrer. A dispersion then purposely warmed to 40°C. for only 5 minutes lost 40% of its sulfhydryl content. Glutathione solutions also show a decrease in sulfhydryl content at room temperature, although the loss appears to be slower than in flour dispersions.

Addition of EDTA also helped to prevent loss of sulfhydryl groups:

	Sulfhydryl Content in Microequiv. per g.
Lee flour	
Commercial urea, no EDTA	0.85
Purified urea, no EDTA	0.91
Commercial urea, 0.001*M* EDTA	0.93
	Equivalents per mole
Glutathione	
Commercial urea, no EDTA	0.77
Purified urea, no EDTA	1.00
Commercial urea, 0.001*M* EDTA	1.02

The catalytic effect of heavy metal ions on the oxidation of sulfhydryl groups is well known; heavy metal ions can also form stable mercaptides. Benesch *et al.* (2) stressed the need for purification of urea and water used in the titrations; however, flour samples themselves will always contribute some heavy metal ions. Chelation of these ions to prevent their bad effects appeared to be a possible alternative to their removal. Ethylenediamine tetraacetate was chosen for trial because of its wide chelating action and ready availability. Addition of the chelating agent to the titration mixtures not only appeared to counteract any effect of metal ions contributed by the flour samples but also allowed the use of unpurified urea, with consequent saving of time and effort required for its purification.

Reproducibility of Results. To minimize the effect of any nonproportionality between titration values and sample sizes, a wide range of

the latter was used (within the limits specified in the method) and the results were calculated by the method of least squares (8). Standard deviations for application of the above procedure to flour and flour extracts generally fell within the range of 0.02–0.04 microequivalents of sulfhydryl per g. of flour for titration values corresponding to 0.6–1.3 microequivalents sulfhydryl per g. of flour.

Specificity of the Method. Benesch *et al.* (2) found that the silver-tris titration was specific for sulfhydryl groups when applied to a variety of amino acids, proteins, and nucleic acids. For flour, the possibility of reaction with or adsorption of silver ion by other constituents, e.g., carbohydrates or lipids, also must be considered. However, removal or blocking of sulfhydryl groups in three different ways prior to titration indicated that consumption of silver ion other than by sulfhydryl groups does not occur. The observations are summarized in Table I. In flour samples stirred in the buffer-urea titration mixture and

TABLE I

EFFECT OF OXIDATION AND MERCURIC CHLORIDE ON THE SULFHYDRYL CONTENT OF FLOURS, STARCHES, GLUTEN, AND A SOLUBLE FRACTION

SAMPLE	TREATMENT BEFORE TITRATION	SULFHYDRYL CONTENT
		microequiv/g
Hard red spring flour	Flour-buffer-urea suspension mixed in air for 27 hours	0.03 [a]
Prime starch	None	None
Lee flour starch (+0.5% protein)	None	0.07 [a]
Lee flour gluten	Sample was oxidized for 20 hours at 8°C. with performic acid	None [a]
Lee flour solubles	Sample was oxidized for 7 hours at 4°C. with performic acid	None [a]
Hard red spring flour	3:1 equivalents Hg++/-SH; 25 minutes reaction at 25°C.	None
Cookie flour	4:1 equivalents Hg++/-SH; 25 minutes reaction at 25°C.	None

[a] Calculated from varying sample weights by the method of least squares.

exposed to air, the silver nitrate titer was lowered to a nonsignificant value. After separation of a flour into a starch fraction (containing tailings, starch, and 0.5% protein), solubles, and gluten, the starch gave a very small titration; and oxidation of solubles and gluten with performic acid eliminated all silver-binding by these fractions. Finally, in the presence of a three- or fourfold excess of mercuric ion over sulfhydryl, the silver titration was completely eliminated.

Sulfhydryl in Enzyme Digests. The maximum sulfhydryl values obtained for trypsin digests of flours were only about one-third as large as those for flour dispersions. Losses of this order occurred consistently in trypsin digests even though precautions were taken to remove and

exclude oxygen from the samples and digests, and EDTA was added. These observations were made with overnight digestion periods, however, and a decrease in initial rate of loss by the treatments mentioned could have occurred undetected. The possibility of reaction between sulfhydryls and some other flour constituents, e.g. methoxy-*p*-benzoquinone (3), has not been investigated, but such reaction may account to some extent for the poor recovery of sulfhydryl in the digests.

As mentioned in the introduction, the recovery of sulfhydryl groups from bovine serum albumin in trypsin digests had been high enough (70–90%) to indicate that most of the sulfhydryl groups present in flour were being detected (6). The results reported in the present paper suggest, however, that many of the sulfhydryl groups of flour may be more labile than those of bovine serum albumin. Consequently, the recovery of sulfhydryl groups from added protein, at least in the case of bovine serum albumin, should not be used as a criterion for the nondestruction of flour protein sulfhydryl groups.

Sulfhydryl Contents of Flours. The sulfhydryl contents of a series of quite diverse flours are given in Table II. The values obtained range from 0.66 to 1.30 microequivalents per g. of flour; calculated to a protein basis, they range from 6.1 to 10.3 microequivalents per g. Results on too few samples are available to determine any trends attributable to wheat type, etc.

TABLE II

AVERAGE SULFHYDRYL TITRATION VALUES[a] OF FLOURS

FLOUR	SULFHYDRYL CONTENT	
	microequiv/g flour	*microequiv/g protein*
Lee	0.92 ± 0.02[b]	6.1
Cookie	0.69 ± 0.04	10.3
Hard red spring	1.05 ± 0.05	8.1
Elgin	0.74 ± 0.03	6.2
Idaed	0.87 ± 0.03	7.0
Cracker	0.78	7.8
Fifth break	1.30 ± 0.09	...
Second low grade	1.11	...
Second middlings	0.66 ± 0.04	...

[a] Titration mixtures were 0.001*M* in EDTA only for Lee, HRS, and Elgin flours.
[b] Standard deviation.

The flour results listed in Table II are in the same range as the sulfhydryl values for flours obtained by Baker, Parker, and Mize (1), who employed a ferricyanide oxidation procedure. The results in the present paper also are much closer than those determined on trypsin digests (6) to the values reported by Holme and Spencer (4), but are still somewhat lower. Even slight oxidation of sulfhydryl groups be-

yond the disulfide stage or oxidation of other flour constituents, as may occur in the presence of excess *o*-iodosobenzoate (6), could account for the small difference. Nearly all other reports in the literature related to sulfhydryl contents of wheat products have been concerned only with flour protein preparations.

Some trial experiments have shown that with the analytical method described above and with fractionations conducted at 2°C., flour may be separated into buffer-soluble and -insoluble portions with satisfactory recovery of sulfhydryl groups. The distribution of sulfhydryls in different flour fractions and the reactivity of sulfhydryl groups with various reagents is now being investigated.

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EFFECTS OF SULFHYDRYL-BLOCKING REAGENTS ON THE MIXING CHARACTERISTICS OF DOUGHS[1]

D. K. MECHAM

ABSTRACT

In doughs of an unbleached hard red spring wheat flour, the course of dough formation, development, and breakdown as indicated by recording dough mixers was markedly altered by addition of specific sulfhydryl-blocking reagents (*N*-ethylmaleimide, *p*-chloromercuribenzoate, iodoacetamide) in amounts roughly equivalent to the sulfhydryl content of the flour. When the conventional mixing procedure was used, the reagents shortened time to maximum resistance, slightly increased maximum resistance, and greatly increased the rate of breakdown after the maximum. The changes in mixing curves were larger than were obtained by addition of potassium iodate and sodium chlorite.

To determine the effects of reagent concentration and reaction time, a modified procedure was used in which flour, water, and reagents were incorporated into doughs with a minimum of hand-mixing before mixing curves were obtained. With control doughs, the mixing time to maximum resistance decreased from 5.2 minutes with no standing time to 1.0 minute after 6 hours. With doughs containing sulfhydryl-blocking reagents in amounts equivalent to from one-half to three times the flour sulfhydryl content, maximum resistance was reached in 3.0 to 4.0 minutes regardless of standing time up to 6 hours. The rate of breakdown of the treated doughs also was unaffected by standing time and was much faster than that of control doughs.

Similar effects were observed with unbleached short-mixing-time flours and a bleached and bromated baker's patent flour. No change in rate of destruction of yellow pigments during mixing was observed when *N*-ethylmaleimide was added to doughs.

The extremely varied dough-mixing characteristics of flours from different wheat types, varieties, etc., have prompted several studies of mixing properties in relation to flour composition and dough treatment.

Miller and co-workers (11) found, in a comparison of composite samples of five hard red winter wheat varieties grown in two crop years, that the cystine-plus-cysteine contents of whole wheats and the mixing times of the corresponding flours were consistently higher in one of the crop years. No other relationships with reactive groups of flour proteins have yet been suggested. However, Mattern and Sandstedt (10) reported observations that indicate the mixing requirement of a flour is determined principally by water-soluble but gliadinlike constituents. Pence, Elder, and Mecham (12) observed that crude albumin preparations decreased mixing times in reconstituted doughs prepared with wet gluten.

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Among the numerous materials that have been incorporated in doughs and found to modify the curves obtained in recording dough mixers are reducing agents (7,19,22), proteolytic enzymes (20), oxidizing agents (4), and detergents (18,21). That oxygen from the air incorporated in a dough also has an effect was shown by comparisons of mixing curves run in air and nitrogen atmospheres as well as by baking (5,14).

In general, the possibility that protein-sulfhydryl groups may be involved in dough development and mixing stability appears to have been given only secondary consideration. Usually, sulfhydryl groups have been studied in relation to the effects of oxidizing agents on physical properties of resting or fermenting doughs. Goldstein (6), for example, used *p*-chloromercuribenzoate to block sulfhydryl groups in doughs, but reported only effects shown by the Extensograph® and did not refer to mixing curve effects. Smith *et al.* (15,16) reported a decrease in sulfhydryl content of doughs as oxygen is absorbed in dough mixing, but these authors were primarily concerned with changes in sulfhydryl content as related to dough properties measured by the Extensograph.

The effects on dough mixing curves of reagents that are generally considered to react more specifically with sulfhydryl groups than oxygen or oxidizing salts strongly suggests that sulfhydryl groups do play a governing role in dough mixing behavior. Observations from such experiments are described in this paper. In addition, an interpretation of the results is presented to suggest that sulfhydryl-disulfide interchange may occur during mixing.

Materials and Methods

The flour designated "HRS" was a commercially milled unbleached flour, from Montana wheats, containing 12.7% protein and 0.25% ash, 14% moisture basis. The flour designated "Baker's Patent" was a commercially milled, bleached and bromated bread flour containing 12.4% protein and 0.44% ash, 14% moisture basis. The Elgin and Idaed flours were experimentally milled flours from pure-variety lots of wheat; protein and ash contents were, respectively, 11.9 and 0.56% for the Elgin, and 12.4 and 0.52% for the Idaed. Elgin is a white club variety; Idaed is a white spring common wheat variety.

The *N*-ethylmaleimide (NEMI), *p*-chloromercuribenzoate (PCMB), potassium iodate, and sodium chlorite were commercial products. The iodoacetamide was a laboratory preparation. These reagents, as well as sodium chloride when included in the doughs, normally were dissolved in a portion of the water before they were added to doughs.

Farinograph curves were obtained with a small bowl (50 g. flour, 14% moisture basis). The conventional mixing procedure was employed with some doughs. With others, a hand mixing procedure was used in which water, reagent solution, and flour were mixed with a spatula in a bowl only long enough to distribute liquid fairly uniformly throughout the dough, as judged by its appearance. Thirty seconds or less were required in most cases. Hand-mixed doughs that were not transferred immediately to the farinograph bowl were let stand at room temperature, about 25°C., with the bowl covered.

Sulfhydryl determinations were made by the procedure described by Sokol *et al.* (17). Water-saturated *n*-butyl alcohol was used for pigment extractions (2). The physical properties of the doughs, especially those that became badly overmixed, made their disintegration and extraction difficult; also, the extracts often were turbid. Reproducible results consequently were not obtained until the following procedure was adopted. Doughs were mixed from 40 g. flour for the desired time in a micro-recording mixer (National Mfg. Co., Lincoln, Nebraska).[2] Then 5 ml. of saturated potassium sulfate solution were added to the dough and incorporated into it by an additional minute of mixing. The added salt made the doughs less sticky and stringy. Most of the dough (weighed by difference to 0.5 g.) was placed in a 1-pt. Mason jar, 100 ml. water-saturated *n*-butyl alcohol were added, and the jar was closed with an Osterizer cap (John Oster Mfg. Co., Racine, Wis.) containing a blending and disintegrating attachment. The dough samples were dispersed by use of the blender, and treatments were repeated at least three times for 1 minute each at 1-hour intervals and again after overnight standing. Flour contrlos were run in which water and the salt solution were added with the alcohol to the flour just prior to blender treatment. The extracts were clarified by centrifuging, and their optical densities were compared in a Coleman Model 6A colorimeter, using 25 mm.-diameter round cuvets and a wave-length setting of 436 mμ.

Results

The marked alteration of farinograph curves that may be produced by small amounts of NEMI is illustrated in Fig. 1; the curves were obtained by the conventional mixing procedure with HRS flour. The largest addition of NEMI, 16 mg., was equivalent to about 2.4 times the sulfhydryl content of the flour.

As the amount of NEMI was increased, the maximum resistance to

[2] Mention of trade names or equipment does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

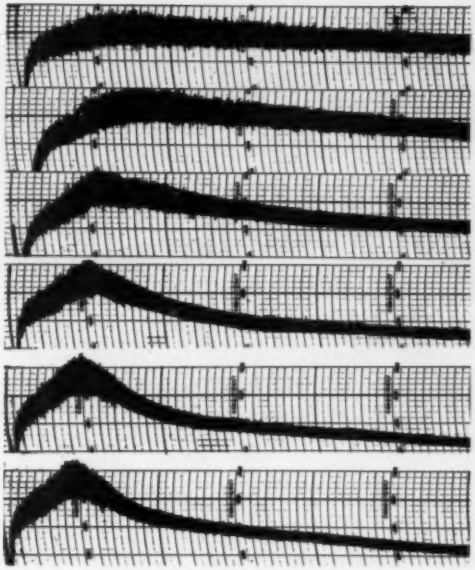

Fig. 1. Effect of *N*-ethylmaleimide on farinograph mixing curves. All doughs contained 50 g. HRS flour on a 14% moisture basis, 0.75 g. sodium chloride, and 30.3 ml. water. The amounts of *N*-ethylmaleimide in the doughs were, from top curve to bottom curve, 0, 1, 2, 4, 8, and 16 mg., respectively.

mixing (at constant absorption) increased, the time to reach the maximum decreased, and the rate of breakdown beyond the maximum increased greatly. Because these effects were so pronounced and because the sulfhydryl content of flours in general is rather low (1.05 microequiv. per g. of HRS flour), there was a question whether the effects could be attributed to sulfhydryl blocking. Further information therefore was obtained, as reported below.

Effects of Other Sulfhydryl-Blocking Reagents. The specificity of *N*-ethylmaleimide for reaction with sulfhydryl groups is well established for isolated proteins and amino acids (23), but no evidence is available regarding its specificity in a material such as flour. Iodoacetamide and PCMB also have been used as reagents specific for sulfhydryl groups in proteins (14). To determine whether these two reagents, and mercuric chloride as well, would affect farinograph mixing curves in the same way as NEMI, the curves in Fig. 2 were made. Only results with HRS flour are presented, but similar results were obtained with the other flours. The amount of reagent added was not varied systematically, but in each case it was more than equivalent to the total sulf-

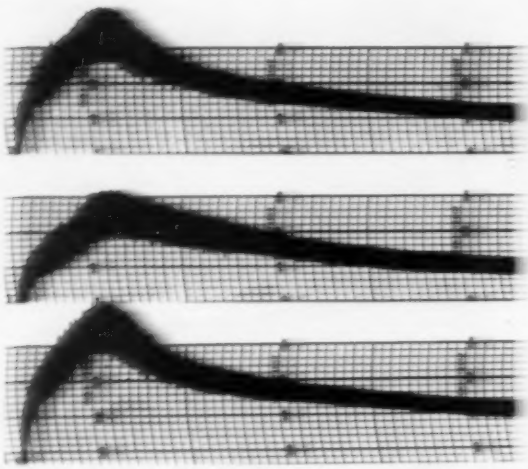

Fig. 2. Effects of other sulfhydryl-blocking reagents on farinograph mixing curves. All doughs contained 50 g. HRS flour on a 14% moisture basis, 0.75 g. sodium chloride, and 30.3 ml. water. Top curve, 30 mg. *p*-chloromercuribenzoate; middle curve, 20 mg. iodoacetamide; bottom curve, 20 mg. mercuric chloride. These amounts were equivalent to about 1.5, 2.0, and 1.5 times, respectively, the total sulfhydryl content of the flour.

hydryl content of the flour. In general, these reagents modify the mixing curve very much as NEMI does. The effects of PCMB and mercuric chloride on maximum resistance are larger relative to their effects on time to maximum and rate of breakdown than those of NEMI; but the similar effects of all the reagents makes reaction with sulfhydryl groups seem the most probable basis for the principal effects noted.

TABLE I

Effects of Rapid-Acting Oxidizing Agents on Farinograph Mixing Curves in Comparison with Sulfhydryl-Blocking Reagents

Added Reagent[a]	Mixing Time to Maximum	Resistance[b]	
		Maximum	20 Minutes Past Maximum
	minutes	*BU*	*BU*
None	9.0	520	480
Potassium iodate, 10 mg.	9.0	540	410
Sodium chlorite, 5 mg.	7.5	560	400
Sodium chlorite, 10 mg.	9.0	550	390
Sodium chlorite, 20 mg.	8.2	550	360
N-ethylmaleimide, 8 mg.	4.8	580	350
p-Chloromercuribenzoate, 30 mg.	5.5	630	420
Iodoacetamide, 20 mg.	6.1	550	400

[a] All doughs contained 50 g. HRS flour, 14% moisture basis, 0.75 g. sodium chloride, and 30.3 ml. water.
[b] Read at center of curve band.

Oxidizing Agents. Potassium iodate is known to be a fast-acting improving agent in baking and to modify farinograph mixing curve characteristics in ways quite similar to the effects of NEMI. To provide a direct comparison on the same flour, the results in Table I were obtained. Observations made with sodium chlorite, another fast-acting oxidizing agent, also are given. Although these oxidizing agents did modify the curves obtained, the changes were not so marked as those produced by NEMI and the other specific sulfhydryl reagents, especially if the higher dosage of oxidizing agents (in microequiv. per g. flour) is considered.

Comparisons of Reagent Levels and Reaction Times. Despite the qualitatively similar effects of the various sulfhydryl reagents, the progressively larger changes in mixing characteristics of the HRS flour as NEMI additions were increased (Fig. 1) did not appear consistent with the changes being attributed solely to sulfhydryl blocking. The total sulfhydryl content of the flour would require only 6.6 mg. NEMI for complete blocking, yet in Fig. 1 at least twice that amount was required to reach the maximum effect. In the absence of information on the extent and rate of reaction of the flour sulfhydryl groups, the observations suggested that other groups were reacting or that adsorption of reagent might account for some effects.

The hand mixing procedure was adopted to obtain curves that

TABLE II

EFFECTS OF SULFHYDRYL-BLOCKING REAGENTS ON FARINOGRAPH CURVES OF HAND-MIXED DOUGHS

Added Reagent[a]	Standing Time	Mixing Time to Maximum	Resistance[b]	
			Maximum	20 Minutes Past Maximum
	hours	*minutes*	*BU*	*BU*
None	0	5.2	570	485
	1	2.2	495	410
	3	1.0	500	410
	6	1.0	460	350
N-ethylmaleimide, 3.3 mg.	0	3.5	560	320
	1	3.4	490	295
	3	3.4	485	310
	6	3.1	470	305
N-ethylmaleimide, 19.8 mg.	0	3.9	560	270
	1	3.8	485	250
	6	3.8	480	255
Iodoacetamide, 9.8 mg.	0	3.9	540	330
	3	3.4	465	310
Iodoacetamide, 29.4 mg.	0	4.0	525	320
	3	4.0	480	330

[a] All doughs contained 50 g. HRS flour, 14% moisture basis, and 31.7 ml. water.
[b] Read at center of curve band.

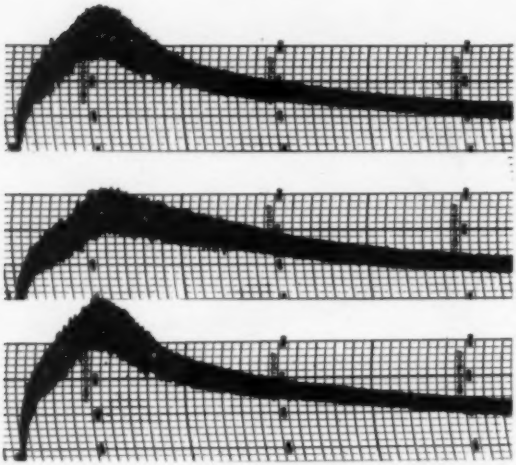

Fig. 2. Effects of other sulfhydryl-blocking reagents on farinograph mixing curves. All doughs contained 50 g. HRS flour on a 14% moisture basis, 0.75 g. sodium chloride, and 30.3 ml. water. Top curve, 30 mg. *p*-chloromercuribenzoate; middle curve, 20 mg. iodoacetamide; bottom curve, 20 mg. mercuric chloride. These amounts were equivalent to about 1.5, 2.0, and 1.5 times, respectively, the total sulfhydryl content of the flour.

hydryl content of the flour. In general, these reagents modify the mixing curve very much as NEMI does. The effects of PCMB and mercuric chloride on maximum resistance are larger relative to their effects on time to maximum and rate of breakdown than those of NEMI; but the similar effects of all the reagents makes reaction with sulfhydryl groups seem the most probable basis for the principal effects noted.

TABLE I

EFFECTS OF RAPID-ACTING OXIDIZING AGENTS ON FARINOGRAPH MIXING CURVES IN COMPARISON WITH SULFHYDRYL-BLOCKING REAGENTS

ADDED REAGENT [a]	MIXING TIME TO MAXIMUM	RESISTANCE [b]	
		Maximum	20 Minutes Past Maximum
	minutes	*BU*	*BU*
None	9.0	520	480
Potassium iodate, 10 mg.	9.0	540	410
Sodium chlorite, 5 mg.	7.5	560	400
Sodium chlorite, 10 mg.	9.0	550	390
Sodium chlorite, 20 mg.	8.2	550	360
N-ethylmaleimide, 8 mg.	4.8	580	350
p-Chloromercuribenzoate, 30 mg.	5.5	630	420
Iodoacetamide, 20 mg.	6.1	550	400

[a] All doughs contained 50 g. HRS flour, 14% moisture basis, 0.75 g. sodium chloride, and 30.3 ml. water.
[b] Read at center of curve band.

Oxidizing Agents. Potassium iodate is known to be a fast-acting improving agent in baking and to modify farinograph mixing curve characteristics in ways quite similar to the effects of NEMI. To provide a direct comparison on the same flour, the results in Table I were obtained. Observations made with sodium chlorite, another fast-acting oxidizing agent, also are given. Although these oxidizing agents did modify the curves obtained, the changes were not so marked as those produced by NEMI and the other specific sulfhydryl reagents, especially if the higher dosage of oxidizing agents (in microequiv. per g. flour) is considered.

Comparisons of Reagent Levels and Reaction Times. Despite the qualitatively similar effects of the various sulfhydryl reagents, the progressively larger changes in mixing characteristics of the HRS flour as NEMI additions were increased (Fig. 1) did not appear consistent with the changes being attributed solely to sulfhydryl blocking. The total sulfhydryl content of the flour would require only 6.6 mg. NEMI for complete blocking, yet in Fig. 1 at least twice that amount was required to reach the maximum effect. In the absence of information on the extent and rate of reaction of the flour sulfhydryl groups, the observations suggested that other groups were reacting or that adsorption of reagent might account for some effects.

The hand mixing procedure was adopted to obtain curves that

TABLE II

EFFECTS OF SULFHYDRYL-BLOCKING REAGENTS ON FARINOGRAPH CURVES OF HAND-MIXED DOUGHS

Added Reagent[a]	Standing Time	Mixing Time to Maximum	Resistance[b]	
			Maximum	20 Minutes Past Maximum
	hours	*minutes*	*BU*	*BU*
None	0	5.2	570	485
	1	2.2	495	410
	3	1.0	500	410
	6	1.0	460	350
N-ethylmaleimide, 3.3 mg.	0	3.5	560	320
	1	3.4	490	295
	3	3.4	485	310
	6	3.1	470	305
N-ethylmaleimide, 19.8 mg.	0	3.9	560	270
	1	3.8	485	250
	6	3.8	480	255
Iodoacetamide, 9.8 mg.	0	3.9	540	330
	3	3.4	465	310
Iodoacetamide, 29.4 mg.	0	4.0	525	320
	3	4.0	480	330

[a] All doughs contained 50 g. HRS flour, 14% moisture basis, and 31.7 ml. water.
[b] Read at center of curve band.

could be interpreted more straightforwardly. When hand mixing is used, sulfhydryl groups may react with the added reagent before development begins, and doughs can be permitted different reaction times to allow additives to exert their full effect before comparison of doughs is made in the farinograph.

When such a procedure was used with the HRS flour, a marked change occurred in the control doughs as they were allowed to stand (Table II); their development time decreased from 5.2 to 1.0 minute, maximum resistance decreased, and resistance at 20 minutes past the maximum decreased. However, with the addition of 3.3 mg. NEMI (equivalent to one-half the sulfhydryl content of the flour), development time was stabilized to a marked degree, and maximum resistance was reached within 3.0 to 4.0 minutes of mixing. Additions of six times this amount of NEMI, and of two and six times the equivalent amount of iodoacetamide, also stabilized development time within the same range. The decrease in resistance past the maximum was more rapid in the presence of sulfhydryl-blocking reagents, and the final value (20 minutes past maximum) was much less affected by time of standing than in the absence of reagent. The approximately equal effects of NEMI and iodoacetamide over a sixfold range in levels of addition and over a 6-hour period are difficult to account for except as the result of sulfhydryl blocking.

Curves also were obtained with 2 mg. NEMI per 50 g. HRS flour. This amount of NEMI was not quite sufficient to stabilize the dough, as shown by a decline in mixing time to reach maximum resistance as standing time was extended; values were 3.5, 3.1, 3.0, and 2.4 minutes at 0, 1, 3, and 6 hours.

Behavior of Other Flours. The effects of NEMI on the mixing behavior of flours of quite different types were found to be qualitatively similar. With short-mixing-time flours the decrease in time to peak was, of course, much smaller, but the breakdown of flours of poor mixing stability was made even more rapid. Results with two flours of this type are summarized in Table III.

NEMI additions to doughs prepared from a bleached and bromated flour caused changes in mixing curve characteristics similar to those observed with the unbleached flours (Fig. 3). A point of interest here was that the control doughs changed in mixing properties on standing even though the flour was bleached and bromated; the stabilizing effect of NEMI thus appears to be distinct from the action of the bleaching agent and bromate. Potassium iodate appeared to stabilize doughs of HRS flour in a limited trial, but the reduction in mixing time was not so great as given by NEMI. Mixing times of 5.0, 4.5, and 5.0 min-

TABLE III

EFFECT OF N-ETHYLMALEIMIDE ON FARINOGRAPH CURVES OF HAND-MIXED DOUGHS FROM SHORT-MIXING-TIME FLOURS

Dough Treatment [a]		Standing Time	Mixing Time to Maximum	Resistance [b]	
Flour	N-Ethylmaleimide			Maximum [c]	20 Minutes Past Maximum
		hours	*minutes*	*BU*	*BU*
Elgin	None	0	0.5	610	310
		3	0.1	640	285
Elgin	6.6 mg.	0	0.4	640	210
		1	0.4	630	205
		3	0.2	670	210
Idaed	None	0	0.5	565	370
		3	0.3	620	320
Idaed	5.6 mg.	0	1.2	600	245
		3	1.0	520	225

[a] 50 g. flour, 14% moisture basis, with 26.3 ml. water for the Elgin and 27.1 ml. water for the Idaed doughs.

[b] Read at center of curve band.

[c] The maximum value on hand-mixed doughs from short-mixing-time flours was less reproducible than on doughs from long-mixing-time flours.

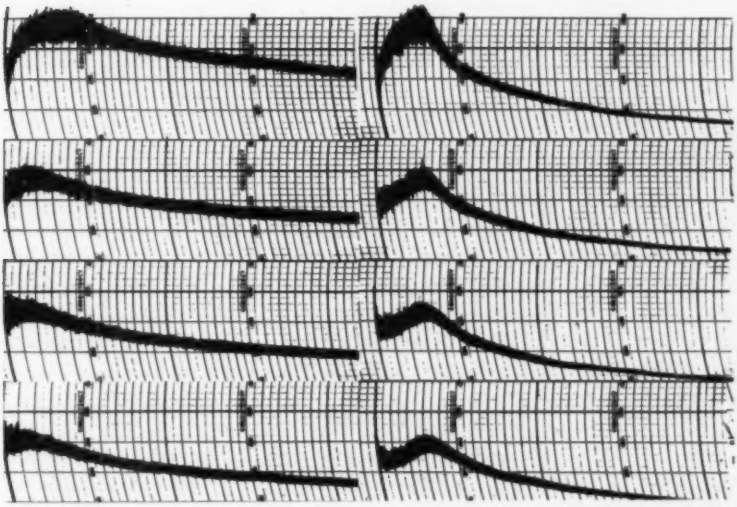

Fig. 3. Farinograph curves on hand-mixed doughs from a bleached and bromated Baker's Patent flour, showing the effects of rest time and of *N*-ethylmaleimide. Curves on the left are from control doughs, hand-mixed, and given (top to bottom) 0, 1, 3, and 6 hours' rest time, respectively. The curves on the right were from doughs given the same treatments but 6.6 mg. NEMI were added to each (6.6 mg/50 g flour). A dough run by the conventional procedure (not shown) required 6.5 minutes to reach its peak; the two top curves in this figure reached their peaks at 4.0 and 2.5 minutes.

utes to peak were required after 0, 1, and 3 hours' standing time when 10 mg. iodate were added to doughs from 50 g. HRS flour.

Lack of Effect on Pigment Destruction. Doughs containing NEMI consistently appeared whiter than control doughs without NEMI when both were mixed an equal length of time. It was suspected that the difference in appearance was a reflection of the different physical state of the NEMI doughs: they had a shiny surface typical of overmixed doughs. On the other hand, if absorption of oxygen from air through a lipoxidase system can lead either to oxidation of flour pigments (9) or to oxidation of –SH groups (16), then blocking of –SH groups might enhance the oxidation of pigments.

Comparisons of the amounts of extractable pigments in control and NEMI-treated doughs therefore were made, with the results given in Table IV. The mixing times were selected to give doughs just prior

TABLE IV

PIGMENT DESTRUCTION IN DOUGHS TREATED WITH N-ETHYLMALEIMIDE

DOUGH TREATMENT[a]		OPTICAL DENSITY OF EXTRACT
N-Ethylmaleimide	Mixing Time	
mg	*minutes*	
None	None (flour)	0.238, 0.249
None	3	0.200, 0.200
4.6 mg.	3	0.197, 0.201
None	6	0.177, 0.178
4.6 mg.	6	0.175, 0.171

[a] 35 g. flour, 14% moisture basis, 22 ml. water.

(at 3 minutes' mixing) to the rapid breakdown that occurred when NEMI was present and (at 6 minutes) well after the NEMI-treated doughs had deteriorated. If pigment concentration is assumed to be directly proportional to optical density of the extracts, then at 3 minutes 18% of the pigment in both samples had been destroyed, and at 6 minutes 27 and 29% had been lost. No indication that destruction of pigments is affected by the presence of NEMI is given by these results.

Discussion

The observations described in the preceding sections indicate that sulfhydryl blocking is responsible for the marked changes in mixing curves produced by the various reagents. No other reasonable explanation is evident for the similar effects of different sulfhydryl reagents and, particularly, for the substantially equal effects produced over a range of reagent concentrations and reaction times.

In general, however, the results provide little evidence as to the mechanisms by which the relatively few sulfhydryl groups in flours

(and probably only a certain fraction of these) exert such a pronounced effect on mixing properties. Such information probably will have to be obtained, for example, by determining the protein fraction containing the affected sulfhydryl groups, by showing whether enzyme inhibition is involved, and by relating the effects of sulfhydryl blocking to those of mixing in air and in oxygen-free atmospheres, as described in the literature (1,5,15,16).

These reports actually prompted the present work. It was thought that if sulfhydryl groups were involved in mixing behavior of doughs, then the differing behavior in air and in oxygen-free atmospheres must arise from oxidation of sulfhydryl to disulfide during mixing in air. Blocking of sulfhydryl groups with NEMI then would prevent this oxidation, and the mixing behavior of NEMI-treated doughs in air should be the same as that of untreated doughs in nitrogen. This was not the effect observed, however; the NEMI caused changes that were more marked than any ascribed in the literature to a change in atmosphere and that were opposite to the enhancement of mixing stability in oxygen-free atmospheres usually reported.

The observations thus suggest that oxidation of sulfhydryl to disulfide is not the principal way in which sulfhydryls are related to mixing behavior. Consequently, other ways in which sulfhydryl groups could be involved need to be considered. The presence of enzyme systems in flour, dependent on sulfhydryls and inactivated by NEMI and the other reagents, must be considered as one possibility. A second possibility is an interchange between sulfhydryl and disulfide groups during dough development.

The latter possibility is especially interesting because such interchanges could build up a network of linked protein molecules throughout a dough. Interchanges of this type have been postulated to explain gel formation in solutions of serum albumin to which a small amount of sulfhydryl compound was added (8); gliadin solutions behaved in a similar way (13). The occurrence of such interchanges also has been proposed to explain changes in the long-range elasticity of wool fibers that are stretched over 30% (3). Goldstein (6) suggested that the mixing of doughs provides a somewhat parallel case, and pointed out that the likelihood for a sulfhydryl and disulfide group to come into contact is probably greater than that for two sulfhydryl groups, because of the much greater numbers of disulfide groups in gluten. He apparently considered that sulfhydryl-disulfide interchange would produce only deteriorative changes in the dough protein system, however.

Assuming that sulfhydryl-disulfide interchange reactions do occur in dough mixing, then one might speculate briefly on their relation

to the dough mixing curve. The development and stable portions of the mixing curve may represent periods during which a protein network is being formed and there are sufficient sulfhydryl groups present to prevent the building-up of severe strains in the dough. As mixing continues, however, the network becomes more nearly continuous and offers more resistance to mixing, and few sulfhydryl groups are present because many have been oxidized. Little interchange then can occur to relieve strains within the doughs, and breakdown of the dough's resistance to mixing then may begin. Blocking of sulfhydryl groups to prevent all interchanges would cause these changes in doughs to take place more rapidly. At the present time, however, the interchange reaction itself has not been demonstrated to occur during dough mixing, and other possibilities must also be considered in future work.

Acknowledgments

The technical assistance of Mr. Max Hanamoto and Mrs. Shirley Knobloch in carrying out this work is greatly appreciated.

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EFFECT OF TEMPERATURE ON THE KINETICS OF THE LIPOXIDASE SYSTEM OF WHEAT[1]

G. N. IRVINE

ABSTRACT

Kinetic studies of the oxidation of linoleic acid by lipoxidase extracts from wheat show that oxygen uptake occurs in three phases: the first is a rapid zero-order reaction which very shortly decays (transition phase) to a steady state phase representing a slower zero-order reaction. The effect of temperature on the kinetics of the two main phases has been studied in the range 0° to 50°C. Arrhenius plots for the initial phase show a distinct break in the region of 15° to 20°C. with activation energies of 8.2 kcal. below this range and 3.2 kcal. above it. The initial phase is of short duration above 40°C. The steady state phase shows a similar break in the activation energy plot between 15° and 20°C. The activation energy below 15°C. is 11.7 kcal. and above 20°C. is 5.4 kcal. The Michaelis constant for the initial phase also changes abruptly in the region of 15°C.; it decreases rapidly from 0.4° to 15°C. and then increases gradually above this temperature. It is suggested that the initial phase is catalyzed by the free enzyme and the final phase by an enzyme-product complex.

Earlier studies in this laboratory (4) have shown that oxidation of lineoleic acid, when catalyzed by crude extracts of wheat lipoxidase, is a three-phased reaction (under all conditions other than high enzyme and low substrate concentrations); first, a rapid zero-order uptake of oxygen; second, a transition phase in which the rate falls off gradually; and ultimately, a stable rate, again of zero-order, with oxygen uptake of about one-tenth that of the initial zero-order phase.

The ratio of the rates of the initial and final zero-order phases has been found to vary with temperature, with enzyme-substrate ratio, and with total fluid volume of the system. The final zero-order phase is established before the oxygen uptake reaches one mole per mole of substrate and has been followed beyond an uptake of two moles per mole of substrate. Temperature has a profound effect on the over-all reaction: higher temperatures increase the rate of the initial zero-order phase, increase the rate of the transitional phase, and increase the rate of the final zero-order phase; each of the phases, however, has a different activation energy, and hence the change of rate with temperature is different for each one.

With high enzyme and low substrate concentrations, oxygen uptake follows first-order kinetics with respect to substrate concentration; oxygen uptake ceases when the ratio reaches one mole of oxygen per mole

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of substrate, which generally takes only a few minutes (4); this general behavior has been previously observed with soybean lipoxidase by Holman and Bergström (3), who suggested that it offers a useful means of determining linoleic acid concentration. In the present investigation it has been found that after an induction period of several hours a further oxygen uptake occurs, probably by autoxidation of the product. The length of the induction period depends on the temperature and on the relative amounts of enzyme and substrate. The free enzyme in the reaction mixture is believed to act as an inhibitor of autoxidation of the product; but the free enzyme is slowly inactivated by the products, and when this reaction is complete, autoxidation commences.

Kinetic studies have now been made of the effects of temperature on the rates of the three phases of the reaction. Results of these experiments, and of others referred to more briefly, lead to a hypothesis which postulates that the free enzyme catalyzes the first zero-order phase, and that an active enzyme-product complex catalyzes the final phase.

Materials and Methods

The source of enzyme was either the Pelissier or Golden Ball variety of durum wheat; extracts and substrate emulsions were prepared as previously described (5), and reaction rates were studied with a Warburg apparatus. Phosphate buffer, pH 6.5, was invariably used, and the atmosphere in the reaction vessels was generally air, but sometimes it was oxygen, nitrogen, or helium. For experiments involving long reaction times or large uptakes of oxygen, large flasks (approx. 50-ml.), sometimes with double side-arms, were used; otherwise, the normal 15-ml. flasks were used.

Results and Discussion

1. The Initial Zero-Order Phase

In the following sections the initial, transition, and final phases are dealt with separately and then discussed in terms of the over-all reaction.

Oxygen Requirement. This phase of the reaction apparently requires a certain critical concentration of oxygen. The reaction occurs more slowly in air than at higher oxygen concentrations (4), and it has now been found that the reaction will not go under an atmosphere of commercial nitrogen (99.7%). This is noteworthy, since the final zero-order phase *will* go under this atmosphere despite the very low partial pressure of oxygen. Tappel (12), working with crude soybean extracts at pH 9, reported a Michaelis constant for oxygen of 43 mm.,

which agreed well with Holman's earlier value of 40 mm. (2). The value determined in the present study is 50 mm., which represents reasonable agreement in view of the different pH level used.

Inactivation by Substrate. If enzyme and substrate are mixed under nitrogen, the enzyme apparently reacts with the substrate to form an *inactive complex*. After about 30 minutes under nitrogen, introduction of oxygen does not cause further uptake of oxygen by the enzyme-substrate system. This behavior suggests that there may be two active sites on the enzyme, either of which will react with the substrate, but one of which has a greater affinity for oxygen than for the substrate. If the oxygen concentration is too low, an inactive complex is formed, presumably by occupation of both active sites by the substrate; but if the oxygen concentration in the system is high enough, an active complex is formed in which one site is occupied by oxygen and one by substrate.

Inactivation by Products. In the absence of unreacted substrate, the enzyme is also slowly inactivated by the product of the reaction. If an excess of enzyme is allowed to react with a low concentration of

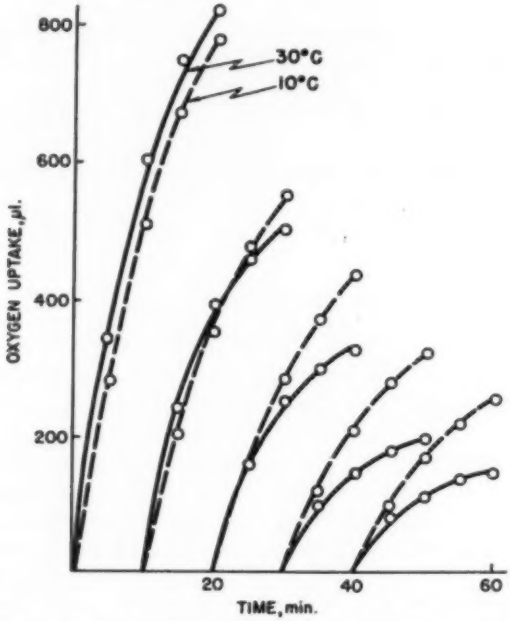

Fig. 1. Oxygen uptake by additions of excess substrate to reaction mixture of enzyme and products after various time intervals at 10°C. and at 30°C.

substrate so that the substrate is all oxidized in about 10 minutes (first-order condition) and if an excess of substrate is subsequently added after a given time interval, then the rate of oxygen uptake following this addition falls off as the interval is increased. This behavior is shown in Fig. 1 for temperatures of 10° and 30°C. The initial rate after addition of excess substrate may be taken as a measure of the active enzyme concentration; a plot of this rate against the time interval shows that inactivation of the enzyme by products is first-order with respect to enzyme concentration. The inactivation is more rapid at 30° C., and the apparent activation energy for this reaction determined from these two temperatures only (hence a very rough estimate) is 4.3 kcal. There is no evidence that this type of inactivation occurs as long as excess substrate is present. These observations suggest that the active sites of the enzyme have a higher affinity for substrate than for products but that, in the absence of substrate, the sites may be occupied by product molecules with a resulting loss of enzymatic activity. There is no evidence to suggest that either inactivation of the enzyme by the substrate or inactivation by the product is reversible.

Variation of Michaelis Constant with Temperature. The initial zero-order rate increases (at a given enzyme level) with increasing substrate concentration up to a maximum, beyond which it is constant in accordance with Michaelis-Menten kinetics; the saturation rate is proportional to enzyme concentration (4). The Michaelis constant was evaluated over the temperature range 0.4° to 35°C. at 5° intervals. Figure 2 shows that the constant decreases sharply but linearly between 0.4° and 10°C.; an abrupt change occurs in the region of 13° C.; and from 15° to 40°C. the constant increases gradually and linearly. This suggests a fundamental change in the mechanism of the reaction.

Owing to the uncertainty in the relation between the Michaelis constant and the equilibrium constant for formation of the enzyme-substrate complex, further discussion is speculative. Massey (7), working with the enzyme fumarase, observed a similar abrupt change in the Michaelis constant with temperature in the region of 19°C. and found that this was dependent on pH. At higher pH levels this discontinuity was not observed. Accordingly, it appears possible that the effect noted in this work is also pH-dependent, and this may help to account for some of the differences observed among various results reported for lipoxidases from many sources, of different degrees of purity, and at different pH levels. The present work was done at pH 6.5, near the physiological level; whereas much of the work undertaken by others, especially on the lipoxidase from soybean, has been done at pH 9, where is it possible to work with homogeneous substrate solutions.

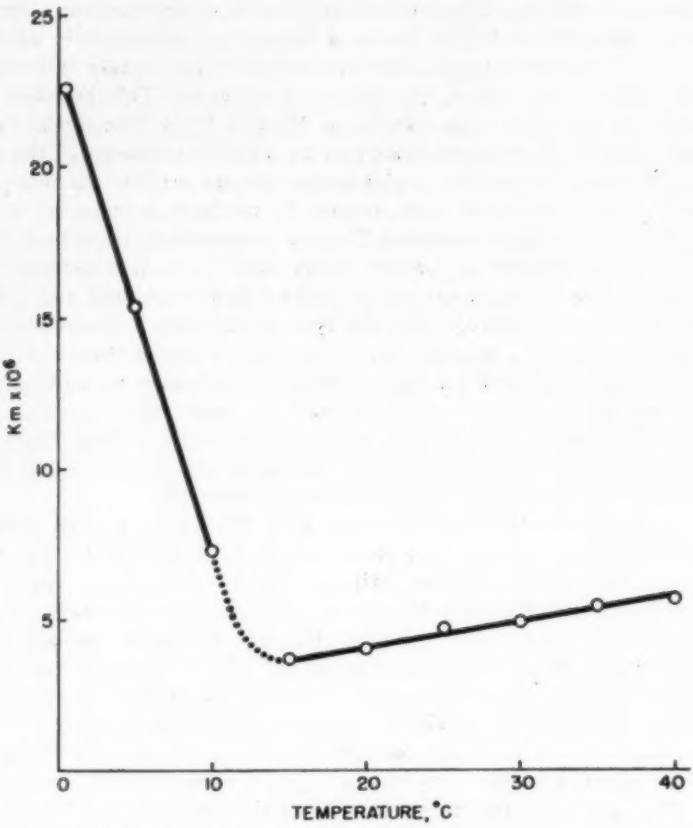

Fig. 2. Plot of Michaelis constant for initial zero-order phase of reaction against temperature.

The Activation Energy. Changes in the rate of the initial zero-order phase with temperature were studied by duplicate measurements of oxygen uptake at 30-second intervals for the first few minutes of reaction over the temperature range from 0° to 35°C.; at temperatures above 35°C. the transition between the initial and final zero-order phases is too rapid to permit an accurate measurement of the initial rate.

A plot of the variation in the log of the rate against the reciprocal of the absolute temperature is shown in Fig. 3. A change in the mechanism of reaction is again suggested (compare Fig. 2) by the distinct

break in the Arrhenius plot; the activation energy changes, in the region of 18°C., from 8.4 to 2.5 kcal.

A single value of 6.5 kcal. had previously been reported (4) for the temperature range 10° to 30°C. The present work represents a more detailed study over a wider temperature range and is therefore considered to be more reliable. Tappel (12) has reported a value of 4.2 kcal. for the initial reaction at pH 9, and he found no evidence of a break in the curve over the range −6° to 30°C. Massey (7) noted a break in the activation energy plot for fumarase that was pH-dependent and that disappeared at pH 7. It appears, from Tappel's result and that of the present study, that the activation energy curve for lipoxidase is also pH-dependent. It is also consistent with the behavior noted by Massey that Tappel's value of 4.2 kcal. at pH 9 falls between the two values found in this study at pH 6.5; Massey found for fumarase that the activation energy value at a pH where no break occurred fell between the values noted at pH levels showing a break in the curve.

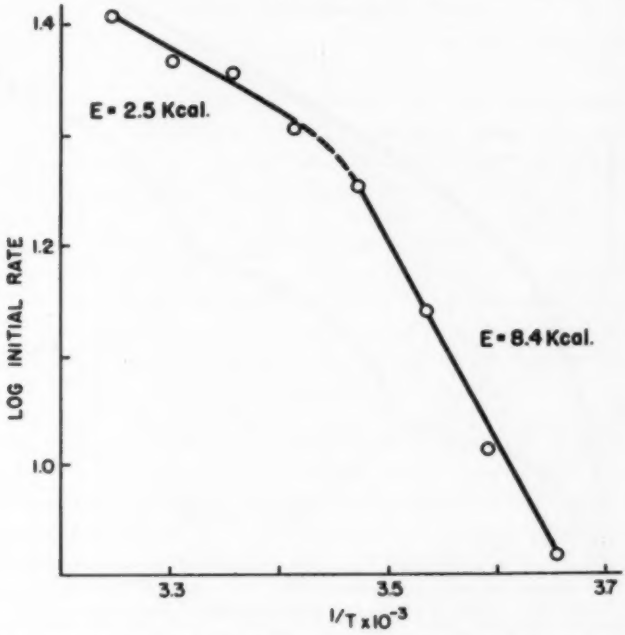

Fig. 3. Activation energy plot for initial reaction rate showing break in activation energy curve.

2. The Transition Phase

The kinetics of this phase of the reaction are ill-defined; it begins as a falling off in the initial zero-order rate and ends with the establishment of the final zero-order rate. Its duration is highly dependent on temperature; at 0°C. it may persist for upwards of 100 minutes, while at 50°C. it persists for only about 5 minutes. Accordingly, at low temperatures the initial and transition phases account for most of the oxygen absorbed unless the experiment is of very long duration; at temperatures above 30°C., the final zero-order phase dominates for reaction times of 3 hours or more. The duration of the transition phase is remarkably independent of enzyme concentration or of amount of substrate when this is in excess, but it appears to depend largely on the concentration of product in the system. It seems probable that the free enzyme of the initial phase reacts with a product to form a less active

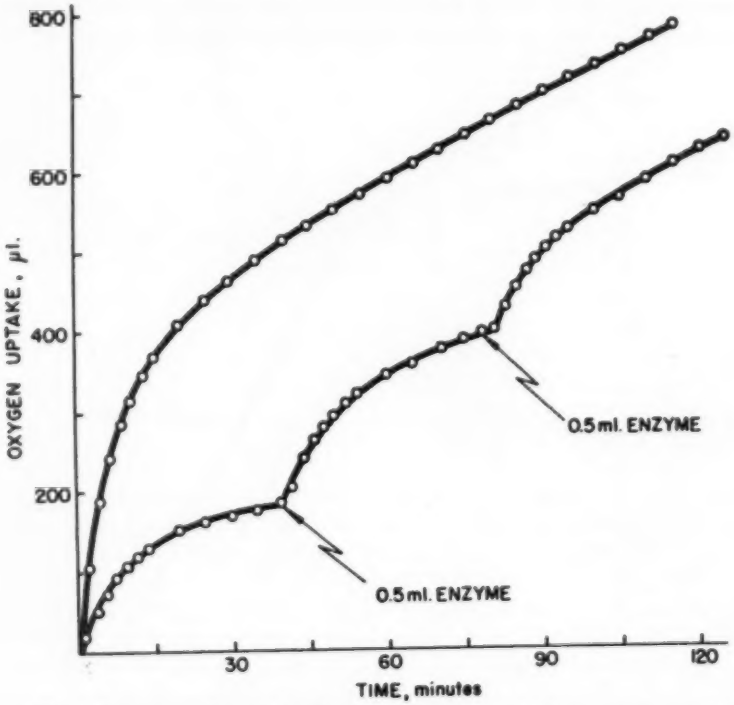

Fig. 4. Oxygen uptake resulting from addition of 1.5 ml. of enzyme at zero time (upper curve) and from addition of increments of 0.5 ml. of enzyme at zero time and after 40 and 80 minutes (lower curve).

complex which is responsible for the final phase of the reaction.

Evidence in favor of this view was obtained in the experiment plotted in Fig. 4. The upper curve shows oxygen uptake for 1.5 ml. of crude enzyme extract. The lower curve shows oxygen uptake for three increments of 0.5 ml. of crude extract tipped in at zero time, 40 minutes, and 80 minutes. The ultimate rate of the final phase, for the experiment in which the enzyme was added in increments, is the same as that for the experiment in which the same total amount of enzyme was added at the beginning of the reaction. The oxygen uptake during the initial rapid phase immediately following addition of an increment of enzyme falls off as the product accumulates in the system. Since there was excess substrate present in both experiments, this fall is not a result of limiting substrate concentration, but appears rather to be the result of a more rapid reaction between the enzyme and the accumulating products of the reaction. Tappel had previously noted a similar effect with soybean lipoxidase (10) but was concerned only with the rate of the initial phase.

3. The Final Zero-Order Phase

Oxygen Requirement. In contrast to the initial phase, the final phase requires only a very low partial pressure of oxygen. It was found that once the reaction had been allowed to proceed through the transition phase, replacement of the air in the flasks with 99.7% nitrogen did not noticeably slow up the rate of reaction. If pure helium replaced the air atmosphere, the reaction did not proceed further.

Inactivation by Substrate. There is no evidence that the enzyme is inactivated by the substrate during the final phase. When the final zero-order rate has been established, further additions of substrate (made via a second side-arm in the Warburg flasks) do not change the rate. There is evidence, however, that the final rate does depend on the initial substrate concentration. It was shown in an earlier paper (4) that, whereas the initial rate increased with increasing substrate concentration only up to a limiting rate (Michaelis-Menten kinetics), the final rate continued to increase within the practical limit of substrate addition.

Inactivation by Products. During the final phase the reaction rate remains constant long enough to account for oxygen uptake of more than two moles of oxygen per mole of substrate. After very long reaction times the rate does fall off eventually, but there is no evidence that this is due to inactivation by products; exhaustion of the substrate appears to be a more probable cause.

Activation Energy. Preliminary experiments suggested that there

was a break in the Arrhenius plot for the final phase in the same temperature range as that found for the initial phase. This was confirmed by careful studies done in duplicate and using long reaction times at the low temperatures to obtain accurate measurements of rates, which were measured at 5° intervals from 0° to 50°C. The break in the plot occurred in the region of 20°C. with activation energies of 11.7 kcal. below this region and 5.4 kcal. above it. This change is in the same direction as that found for the initial zero-order phase (and the change is of the same magnitude), with the lower value being associated with the higher temperature region. However, the actual values of the activation energy are significantly different from those applying to the initial zero-order phase; hence, this final phase of the reaction does not appear to be catalyzed by the same form of the enzyme as is the initial phase.

4. The Over-All Reaction

During the course of this work over one hundred samples of different classes and varieties of wheat have been examined as well as both crude soybean extracts and the crystalline soybean enzyme (General Biochemicals, Inc.). All these show the same three-phase oxygen uptake curve, and, for a given set of reaction conditions, the ratio of the rates of the initial and final phases of the reaction are similar for all preparations.

Most investigators working with lipoxidase extracts have investigated only the initial phase of the reaction and only Süllmann (9), to the writer's knowledge, has observed both the initial and final phases of the reaction as outlined here, and he does not comment on this feature. There is a suggestion of this type of behavior in the data of Holman and Bergström (3) on changes of rate with degree of oxidation at various temperatures, although the method of presentation of the data makes it rather difficult to interpret directly. Craig (1), in some of the earliest work on lipoxidase, used a Warburg apparatus and long reaction times but does not deal with rates. It appears certain, however, that he must have allowed the reaction to proceed well into the final phase. It is perhaps significant that in studies with the Warburg apparatus at long reaction times, presumably involving mainly the final phase, three investigators have reported inhibition by cyanide (1,9,6), although this has not been observed by other workers. Under normal experimental conditions the initial phase is apparently little affected by cyanide, but the final phase is (6); these observations help to account for the lack of agreement on cyanide inhibition between investigators working with different time intervals. The cyanide inhibition

of the final phase suggested that catalase might be involved, since both wheat and soybean extracts contain this enzyme. However, crystalline soybean lipoxidase shows no catalase activity, and as it gives rise to the same oxygen uptake pattern as the crude extracts, this possibility appears to be ruled out.

At present, the available evidence favors the following hypothetical mechanism. The initial zero-order reaction is catalyzed by the free enzyme. During the transition phase, the enzyme forms an enzyme-product complex that is quite stable in the presence of excess substrate, and this complex catalyzes the final zero-order reaction. In terms of oxygen consumed, the complex is less active than the free enzyme. During the transition phase, the enzyme may also form an inactive complex, but this reaction is inhibited by excess substrate.

This general mechanism leaves open the possibility that different substrates and different products are involved during the two stable phases of the over-all reaction. Privett *et al.* (8) studied the products of the lipoxidase-catalyzed oxidation of sodium linoleate and reported that both optically active cis-trans, conjugated monohydroperoxides and optically active polymers are formed, the latter at higher enzyme concentrations and higher temperatures. Holman and Bergström (3) also report differences in the products of reaction at higher temperatures and longer reaction times. This evidence is not inconsistent with the hypothesis that the two phases of the reaction represent different substrates reacting with different forms of the enzyme to yield different products. Thus the products and their relative concentrations would be expected to vary, depending on substrate concentration and temperature employed and also on the duration of the oxidation. The present paper emphasizes the probability that there are at least two active forms of the enzyme: a free form catalyzing the initial phase of the reaction, and a complex form catalyzing the final phase.

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STUDY OF THE STORAGE DETERIORATION AND LOSS OF PERFORMANCE VALUE OF A MILITARY PREPARED CHIFFON CAKE MIX WHEN STORED AT 70° AND 100°F.[1]

J. A. MILLER,[2] C. S. MCWILLIAMS,[2] and J. J. MCMULLEN

ABSTRACT

Storage stability of a chiffon cake mix was studied for a period of one year at 70° and 100°F. Each mix utilized one of four different salad oils: corn, cotton, peanut, and sesame. Duplicate baking tests and chemical analyses were performed, initially and bimonthly, upon the mixes and the separate ingredients. The mixes and individual ingredients were air-packed in hermetically sealed cans.

Prepared mixes have become increasingly popular since World War II, owing in part to their quality and convenience. As a result, an ever-increasing variety is being made available to the consumer. Despite their popularity, prepared mixes have some limitations, the most important of which is their stability. Various workers have described the stability of prepared mixes and the ingredients used in them (4,7,9).[3]

[1] Manuscript received September 23, 1957. Presented at the annual meeting, San Francisco, Calif., May 1957. This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces and has been assigned number 830 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsements of the Department of Defense.

[2] Present addresses, respectively: Victor Chemical Works, Chicago Heights, Illinois; and American Institute of Baking, Chicago, Illinois.

[3] Goss, W. H. The use of shortenings in prepared mixes. Paper presented at a symposium on stability of shortenings in cereal and baked products, sponsored by National Research Council, Advisory Board QM Res. and Dev. Committee on Foods, held in Chicago Jan. 16, 1953.

A chiffon cake mix has been developed by the Quartermaster Food and Container Institute for use in the bakery facilities of the Armed Forces. A chiffon cake is a type of sponge cake. It is dependent on the whipping of egg albumen for production of cake volume, but, unlike a sponge cake, oil is utilized in the batter.

Whole-egg solids have caused a particularly perplexing problem in the production of sponge cakes. Sponge cakes produced from whole-egg solids have poor volume and the batter does not foam as readily as the batters from shell or frozen eggs. Joslin and Proctor (6) found that breakdown of the natural fat emulsion in fresh eggs when dried was the cause of poor foam volume in whipping tests, and that dried whole-egg powder could even cause collapse of foam produced with fresh eggs.

The purpose of the present study was to evaluate the storage stability of military chiffon cake mix in light of adverse storage and climatic conditions that must be considered.

An experimental design was adopted, employing randomization with coding which would leave little chance that any sample would receive preferential treatment.

Baking performance values decreased and organoleptic changes were observed. Chemical analyses of the mixes over 1 year showed slight changes in free fatty acid, moisture, carbonyl, peroxide, carbon dioxide, and pH. Changes in saline fluorescence were more moderate. Substantial changes in lipid fluorescence were observed during storage.

Materials and Methods

Ingredients Employed. A dehydrated, bleached, soft wheat cake flour was used in compounding the chiffon cake mixes. The flour analyzed 5.8% moisture, 8.3% protein ($N \times 5.7$), and 0.45% ash, calculated on an "as-is" moisture basis. The four salad oils, corn, cotton, peanut, and sesame, were deodorized and no antioxidants were added. In addition, the cottonseed oil was winterized. The initial iodine values of the oils were: corn 123.8, cotton 112.4, peanut 93.1, and sesame 113.8. The egg albumen was bacterially treated for removal of reducing sugars, and analyzed 76.93% protein, 7.20% moisture, and less than 0.1% reducing sugars. The whole-egg solids were enzyme-treated for removal of reducing sugars, and analyzed 45.17% protein, 40.68% fat, 4.90% moisture, and less than 0.1% reducing sugars. The baking powder was a commercial pyrophosphate type.

Cake-Mix Formulation and Sample Preparation. The formulations of the mixes are listed on the next page.

The ingredients of "A" mix were uniformly blended together

Cake-Mix Formulation

	Weight	*Total Solids*
	g	*%*
"A" mix		
Egg white solids	50.70	10.14
Sugar	63.35	12.67
Monocalcium phosphate	0.55	0.11
"B" mix		
Sugar	138.35	27.67
Flour	136.20	27.24
Whole-egg solids	54.90	10.98
Salt	3.15	0.63
Baking powder	2.10	0.42
Oil	50.70	10.14
Total	500.00	100.00

in a 30-qt. bowl of a vertical mixer. The "A" mix was then scaled and air-packed in hermetically sealed cans.

The ingredients of "B" mix, with the exception of oil, were blended together in a 60-qt. bowl of a vertical mixer until they were uniform, and then the oil was slowly added to the mix. The machine was stopped and the mix was sifted through a sieve (USS No. 14). The mix was reblended for a short period, then scaled and air-packed in hermetically sealed cans.

The flour, oils, whole-egg, and egg-white solids were individually air-packed in hermetically sealed cans. The "A" and "B" mixes and ingredients were stored at 70° and 100°F. respectively.

Test-Baking and Analytical Methods. Samples were test-baked and chemically analyzed initially and at bimonthly intervals.

Cakes were prepared according to a standardized baking procedure as follows: The "A" mix was reconstituted with water (270 ml.) for 2 minutes at slow speed and then whipped at high speed for 4 minutes in a Hamilton-Beach home-type mixer with large bowl attachment. The "B" mix was mixed with water (110 ml.) on high speed for 2 minutes in the small bowl attachment. Foam from the "A" mix and batter from the "B" mix were then hand-blended until homogeneous. This batter was then scaled at 26 oz. into a 4 by 10-in. tube pan and baked 50 minutes at 350°F.

The baked cakes were immediately inverted on cones for cooling. After cooling for 3 hours, the cakes were removed from their pans and immediately measured for height in millimeters; they were then cut vertically and scored for grain, texture, odor, and flavor. By the scoring procedure used in this study, the height of the cake received 40 points, grain 20 points, texture 20 points, and odor and flavor 10 points each. Cake height score received one point for each mm. above 80 mm. Thus, a cake 80 mm. high would receive a score of zero, 85

mm. a score of 5, etc. Other cake factors were scored subjectively by means of mental conceptions of ideals for each characteristic.

An experimental design was used, employing randomization and coding of the mixes which precluded the possibility that any sample would receive prejudicial treatment.

Moisture content of the mixes and of the individual ingredients was determined by the official AOAC vacuum-oven method (3). Fat was extracted from the mixes and individual ingredients, cold chloroform being used for free fatty acids (3) and peroxide values (2). It is thought that carbonyl values correlate with flavor changes in fats and foods containing fats (5); they were determined in the benzene extractables of the mixes and individual ingredients. Saline and lipid fluorescence were determined as an index of deterioration by the method of Pearce and Thistle (8), modified as to sample size and method of standardization. Saline fluorescence was determined on the mixes and whole-egg solids and lipid fluorescence was determined on the oils, mixes, and whole-egg solids. The pH of the mixes, the several ingredients, and the cake crumb was determined on a slurry by the electrometric method (1). Color of the "B" mixes was measured by the Hunter Color and Color Difference Meter.[4] Total fat content was determined by acid hydrolysis. Total and available carbon dioxide, ash, protein, reducing sugars, and iodine values were determined by methods of the AOAC (3).

Results and Discussion. In designing this test the necessity of performing single or duplicate bakes was questioned. It has been found statistically that it was not necessary to perform the duplicate bakes. The bakes of the first day, on an average, came out slightly higher than those of the second day. This is thought to be a chance effect, in that if the test were repeated the opposite might be the case.

The chiffon cake height data of the cakes, prepared from mixes of each of the four different oils, are seen in Fig. 1. The extremely small differences in the height data between the 70° and 100°F. for the peanut-oil mix sample may indicate better performance stability of this oil in this mix. Of more significance, however, is the very slight effect of storage time and temperature upon cake height or volume.

It should be emphasized that these mixes were compounded with dehydrated cake flour containing less than 6% moisture. Had flour of greater moisture content been used, these data might have been quite different.

We see in Fig. 2 the cake scores plotted against time. As might be

[4] Gardner Laboratories, Bethesda, Md. Description and instructions for Hunter Color and Color-Difference Meter (1950).

expected, there is close similarity in the graphs of the cake height and cake scores, since the heights constitute 40% on the total cake score. There was a correlation of 0.86 between cake height and cake score.

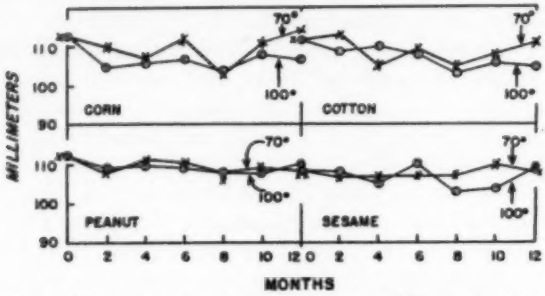

Fig. 1. Effect of storage time and temperature on chiffon cake heights of cakes made from mixes containing one of four different salad oils.

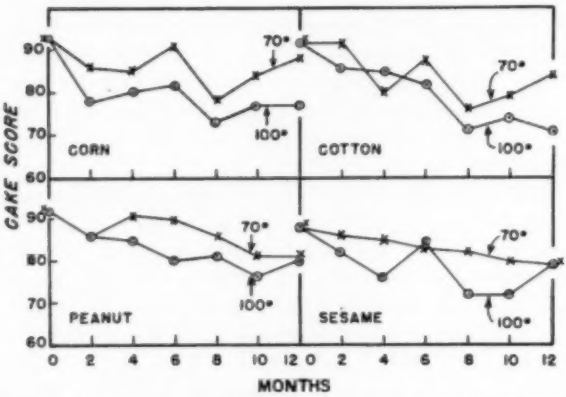

Fig. 2. Effect of storage time and temperature on cake scores of cakes made from mixes containing one of four different salad oils.

There was essentially no difference in the mixes with the various oils or between the storage temperatures, but there was a slight downward trend in the heights and scores over the storage period as analyzed by statistical methods.

Peroxide values of the "B" mixes, containing the various oils, when stored at 70°F., show very little change in comparison with some mixes when stored at 100°F. (Fig. 3). There is one contradiction here, however, in that the "B" mix with cottonseed oil at 100°F. rapidly and consistently developed peroxides.

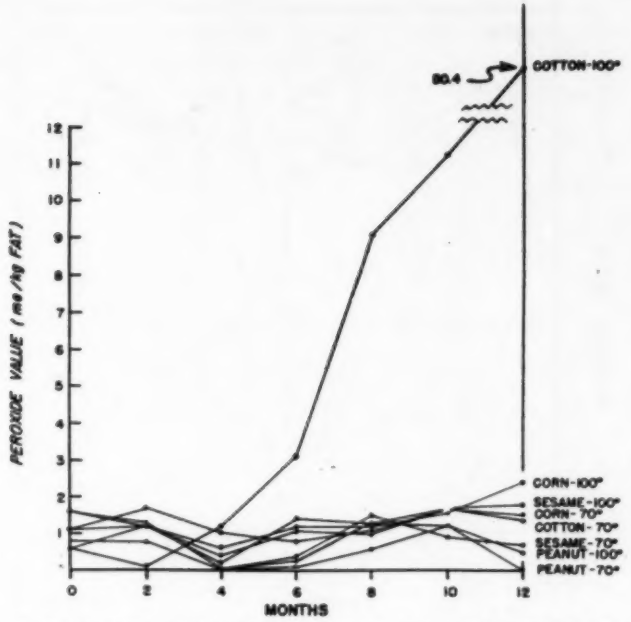

Fig. 3. Effect of storage time and temperature on peroxide values of "B" mixes containing one of four different salad oils.

The peroxide values of the separately stored oils (Fig. 4) increased considerably at both storage temperatures. Unfortunately, the sesame oil was found to have an off-odor that was typical of oxidative rancidity after the mixes were compounded and packaged. Since there is some difficulty in obtaining the dehydrated flour and whole-egg solids, it was decided to continue the testing of this mix even though the sesame oil was oxidized.

The values for the free fatty acids in the "B" mixes are illustrated in Fig. 5 and show only moderate differences between the two temperatures and with time.

Free fatty acids in the oils stored separately increased only very slightly at the two storage temperatures, and the cottonseed had the highest free fatty acid increase. The increase in free fatty acids in the mixes between temperatures and with time is not attributed to the oils.

Free fatty acids of the whole-egg solids are shown in Fig. 6. They show a greater numerical difference between storage temperatures with time than was found in the "B" mixes. The increase in numerical

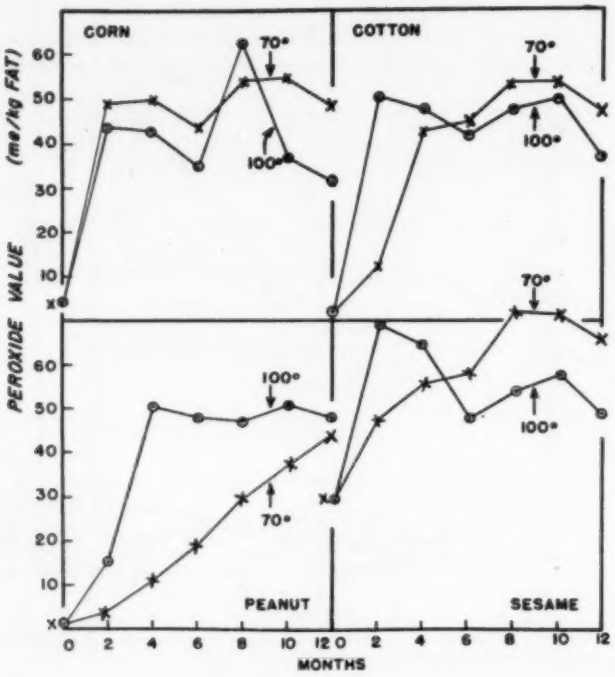

Fig. 4. Effect of storage time and temperature on peroxide values of four different separately stored salad oils used in the chiffon cake mixes.

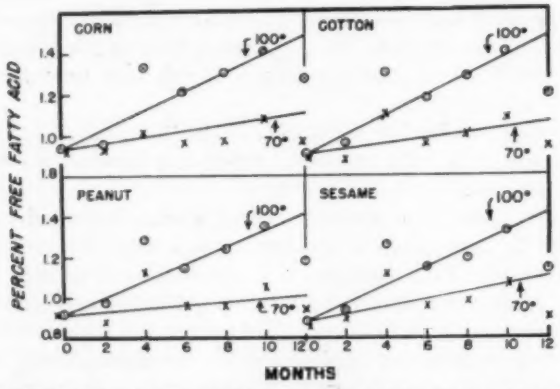

Fig. 5. Effect of storage time and temperature and free fatty acids in "B" mixes containing one of four different salad oils.

differences of free fatty acids between storage temperatures in the "B" mixes is, however, greater when formulation is considered.

The free fatty acids in the flour, as analyzed by chloroform extraction, showed little change between the two temperatures over the

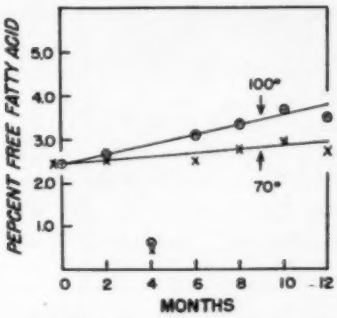

Fig. 6. Effect of storage time and temperature and free fatty acids in the separately stored whole-egg solids.

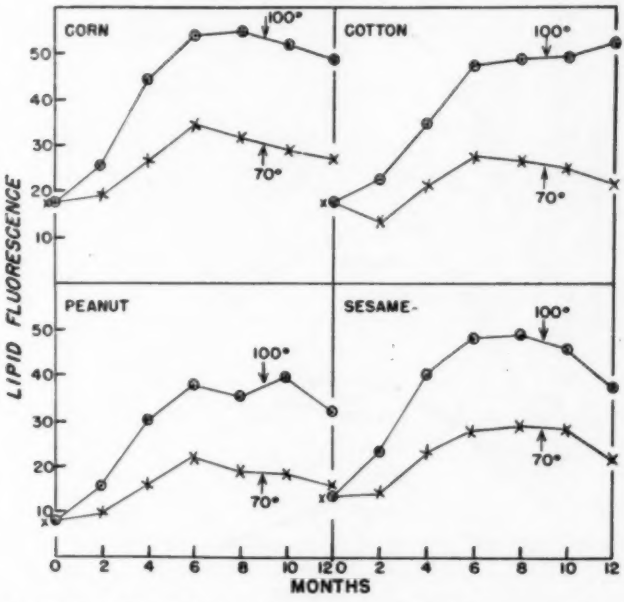

Fig. 7. Effect of storage time and temperature on lipid fluorescence values of the "B" mixes containing one of four different salad oils (microgram equivalent of quinine sulfate per g. of fat).

period of the storage study. This method, however, usually extracts only the unbound fat that is present in the flour.

The values for free fatty acid in the flour and the oils are almost a negligible quantity. It is assumed, therefore, that the increase of free fatty acids in the "B" mixes is dependent on the whole-egg solids, since the values of free fatty acids in the whole-egg solids show the same trend.

Carbonyl values of the oils, "B" mixes, whole-egg solids, and flour showed no significant trends.

The lipid fluorescence values of the "B" mixes showed a moderate increase at 70°F., and a more pronounced increase at 100°F. (Fig. 7).

The values for lipid fluorescence for the separately stored oils are plotted against time in Fig. 8, and show very little deviation between the two storage temperatures. Significant differences are again ob-

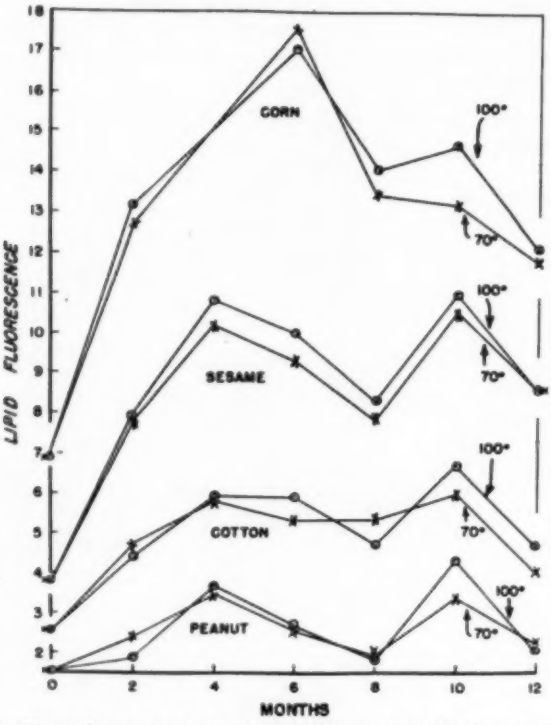

Fig. 8. Effect of storage time and temperature on lipid fluorescence values of four different separately stored salad oils used in the chiffon cake mixes (microgram equivalents of quinine sulfate per g. of fat).

served in lipid fluorescence values for the different oils, with increases following in the order of peanut, cotton, sesame, and greatest in corn.

The lipid fluorescence values of the whole-egg solids are similar

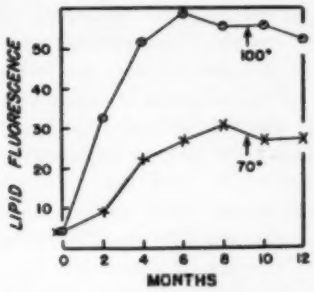

Fig. 9. Effect of storage time and temperature on lipid fluorescence values of separately stored whole-egg solids (microgram equivalent of quinine sulfate per g. of fat).

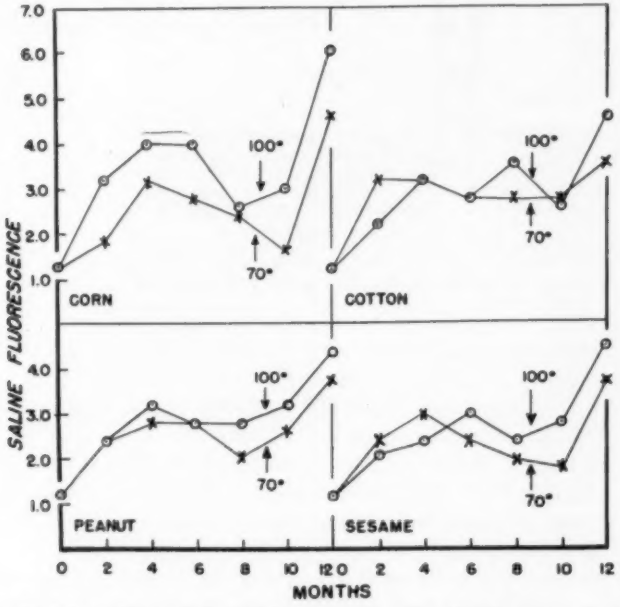

Fig. 10. Effect of storage time and temperature on saline fluorescence values of the "B" mixes containing one of four different salad oils (microgram equivalents of quinine sulfate per g. of fat).

to those of the "B" mixes, as shown in Fig. 9, which shows a slight increase at 70°F., and a larger increase at 100°F.

These results indicate that the whole-egg solids and not the oils are responsible for the divergence of lipid fluorescence values of the "B" mixes stored at 70° and 100°F. The significant differences that were observed in lipid fluorescence values for the different oils are not evident in the "B" mixes to any extent.

Figure 10 illustrates the saline fluorescence values of the "B" mixes over storage time at the two storage temperatures.

Although there was a slight increase in saline fluorescence values in the "B" mixes, the magnitude of these changes differed from the lipid fluorescence values.

The values for saline fluorescence in the whole-egg solids increased, and a divergence between the 70° and 100°F. temperatures can be noted in Fig. 11.

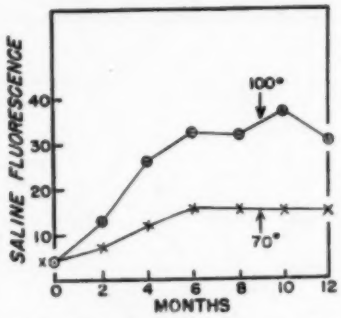

Fig. 11. Effect of storage time and temperature on saline fluorescence values of separately stored whole-egg solids (microgram equivalents of quinine sulfate per g. of fat).

The saline fluorescence values did not show the same quantitative changes shown by the lipid fluorescence values. This leads one to believe that the whole-egg solids did not affect saline fluorescence as they did the lipid fluorescence in the "B" mix.

There was no visible evidence of browning in the "B" mixes, nor did the Hunter Color Difference Meter readings reveal any evidence of browning. The low saline fluorescence values also indicated that browning did not occur.

pH values for the separate mixes and the individual ingredients that were studied decreased slightly as the storage period progressed. The decrease was slightly greater at the higher temperature. pH of the cake crumb showed no changes. Moisture contents of the "A" and

"B" mixes showed no discernible changes. The total, available, and residual carbon dioxide values of the "B" mix showed no significant difference during storage. This indicates that the premature interaction of leavening chemicals was at a minimum.

Some development of an off-odor could be detected upon opening and during blending of the "B" mix near the completion of the storage study. However, any off-flavor or off-odor was not discernible in the finished baked cake.

It can be concluded from these studies that: 1) This chiffon cake mix was found to perform satisfactorily for one year at 70° and 100°F. when air-packed in hermetically sealed cans. 2) Statistical analyses of the cake heights showed no significant difference between the use of single or duplicate bakes. It was shown that neither the type of oil nor the storage temperature caused a significant effect upon the cake height. A slight decrease in baking performance was shown by all cakes after 12 months' storage at 70° and 100°F. 3) There is no essential difference in the performance of the mixes containing the various oils at the two storage temperatures, even though the sesame seed oil was rancid initially and the peroxide values of the "B" mix containing cottonseed oil increased so rapidly.

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A LEAN-FORMULA CAKE METHOD FOR VARIETAL EVALUATION AND RESEARCH[1]

LEO T. KISSELL[2]

ABSTRACT

A simplified lean white layer cake method has been developed for the evaluation of soft wheat varieties. Sensitivity of the cake test is increased by the elimination of egg whites and milk solids, while physical manipulations are reduced to permit mixing at the rate of one sample every 8 minutes. In addition to the differentiation of varieties on the basis of layer volume and internal score, the method is sensitive to differences in liquid-carrying capacities. Sensitivity to other factors, such as 1) extent of bleach treatment, 2) particle size differences, 3) length of patent extraction, and 4) protein content, provides a useful tool for basic research on cake quality.

A sensitive cake test is needed for evaluating new varieties of soft wheat and for basic research on cake quality. Such a test should be: 1) simple enough for application to large numbers of samples, 2) applicable to a small quantity of flour, 3) easily replicable and sensitive to real varietal differences, 4) comparable in results to full-scale commercial test methods, and 5) able to provide information on other cake characteristics in addition to volume and internal score.

Some published high-sugar ratio white layer cake methods have been tried or considered, but none has fulfilled the five basic requirements. The AACC Method (1), the method of Sundberg, Hayashi, and Barmore (5), and the method of Bowman, Maharg, and Poehlman (2) are laborious and time-consuming as routine tests. Also, sensitivity to varietal differences and experimental flour treatments was not sufficiently great when compared with replication errors for each of the methods. A more fundamental approach seemed in order for basic studies on the chemical factors responsible for cake quality as well as routine evaluation of newer wheat selections.

This paper presents a basic formula from which all structure-producing ingredients except patent flour have been excluded. Omission of egg albumen and milk solids should emphasize the structure-producing ability of the test flour and greatly increase sensitivity to varietal and treatment differences.

Materials and Methods

A basic formula containing only flour, sugar, baking powder,

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shortening, and water was tentatively balanced and baked. Resulting cakes were pale in color but satisfactory in volume, crumb structure, and symmetry. Experiments were made to determine optimum conditions for differentiation between a commercial premium cake patent and a low-grade commercial cake flour. A number of ingredient variations were tried including:

1) Dry "Bakers Special" grade sugar alone and in 4:1 ratio with commercial daily whey (4), a commercial 67% liquid sugar (3), and a laboratory-prepared 69% sucrose solution at various levels from 80 to 140% based on flour weight;
2) A commercial double-acting baking powder at levels from 3 to 7%;
3) A commercial high-ratio emulsified shortening at levels from 21 to 35%; and
4) Total liquid as distilled water at various levels from 90 to 138%.

With the criteria of maximum sensitivity and simplicity as objectives, a 69% sucrose solution prepared from standard granulated cane sugar was selected for use in the cake method. The use of dry sugar and sugar-shortening creamed-mass methods involved extra manipulations and did not significantly increase differentiation between test flours. Addition of 20% dairy whey improved crust color but resulted in cakes that were too friable for measurement. Commercial 67% liquid sugar gave satisfactory results but presented procurement and storage problems.

The following "lean-formula cake method" was adopted after the above experiments (and others) to develop mixing and baking methods, as shown in the table below.

Ingredients	*Quantity*	*Composition (Flour Basis)*
		%
Cake flour	150 g.	100
Sugar solution	212 ml.	130[a]
Shortening (high-ratio, emulsified)	41.8 g.	27.9
Baking powder (double action)	7.1 g.	4.7
Distilled water (optimum level is variable among varieties)	55–99 ml.	97–127[b]

[a] Sugar only. Sugar solution prepared by dissolving 3000 g. granulated sugar in 1450 ml. *hot* distilled water. Solution contains 0.92 g. sugar and 0.42 g. water per ml. Specific gravity = 1.340.
[b] Percent total water including water in sugar solution.

Preparation. Flour and baking powder are sifted together. Shortening is weighed on paper squares. Sugar solution is prepared one day in advance so that it and other ingredients are at room temperature (24°–25°C.) before use. Stratification occurs in this concentrated solution as it cools; thorough mixing by a magnetic stirring device is recommended before and during each bake. Since the method is sensitive to

differences in both liquid and sugar content, accurate determination of concentration by specific gravity is essential.

Procedure. Use a Hobart C-100 (or C-10) mixer with 3-qt. bowl and cake paddle. Add flour-baking powder mixture and shortening to the bowl, and dispense sugar solution directly from a 250-ml. automatic buret with a large-bore stopcock.

Mix 0.5 minute at low speed. Scrape down. Mix 2.5 minutes at medium speed. Scrape and then mix for 0.5 minute at medium speed. Add calculated amount of distilled water from automatic buret. Mix 0.5 minute at low speed. Scrape down and mix 1.5 minutes at medium speed.

Divide batter by scaling 240 g. into 6-in. by 1½-in. 4X steel layer pans using 6-in. parchment circles with commercial pan grease on the sides. Bake layers 21 minutes at 375°F. (190°C.) in an electronically controlled reel-type electric oven, conditioned with water vapor before baking begins. Cool the cakes for 15 minutes on wire racks before removal from pans.

Scoring. Volume, contour, and internal appearance are the main criteria used for scoring cakes. Volume is measured by seed displacement approximately 4 hours after baking. A rounded top contour is considered optimum and occurs only at the optimum liquid level for a given flour. Less liquid results in fallen cakes, while excessive amounts produce peaked products. Optimum liquid levels for different samples may vary over a 97–127% range.

Internal appearance is judged in reference to a standard commercial cake patent flour baked each day along with the unknown samples. Cell size, cell-wall thickness, and uniformity of cell distribution are each graded on a 0–5 scale, the summation giving a 0–15 point range from Failure to Excellent. Experience has shown that maximum cake volume, rounded contour, and best internal score usually occur at the optimum liquid level of the sample.

Applications of the Lean Formula

Varietal Differentiation. Extensive testing has indicated that the lean formula ranks varieties in accordance with commercial acceptability when optimum liquid level and optimum chlorine bleach treatment are used.

Varietal differentiation by the lean formula is illustrated with wheat grown at East Lansing, Michigan, in 1955 and 1956. The grain was composited by variety, tempered, and milled on Allis-Chalmers equipment. All samples were milled to a 50% extraction (wheat basis) with break flours taken as throughs of 165-mesh stainless-steel cloth and

TABLE I

ANALYTICAL AND BLEACHING DATA FOR VARIETY PATENTS

VARIETY	ASH[a]	PROTEIN[a]	UNBLEACHED pH	CHLORINE TREATMENT		FINAL pH[b]
	%	%		cc/g	oz/cwt	
Thorne	0.30	7.6	5.7	0.29	1.47	4.7
Clarkan	0.31	8.0	5.8	0.32	1.62	4.8
Trumbull	0.30	8.1	5.8	0.33	1.67	4.8
Purkof	0.35	7.7	6.0	0.38	1.93	4.7

[a] 14% Moisture basis.
[b] Determined 1 week after bleaching.

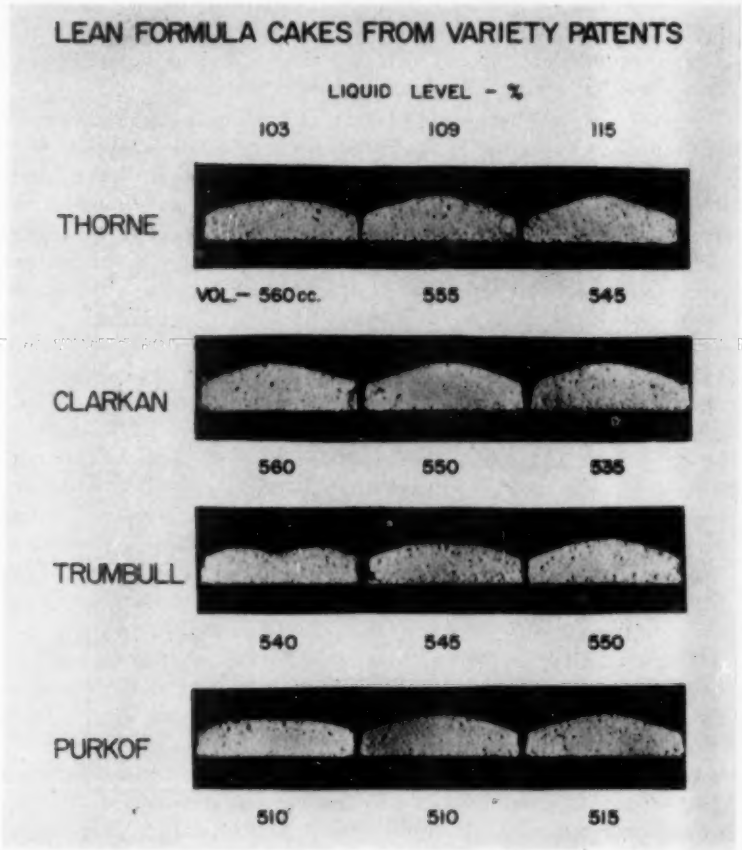

Fig. 1. Cross sections of cakes, showing the effect of liquid content on cake volume and contour.

reductions as throughs of 200-mesh. This experimental milling approximated premium commercial patent flours with low average protein content (8.0%), low ash (0.32%), and relatively fine granulation. Based on experience, a pH range of 4.7–4.8 was chosen as a suitable bleaching level for these varieties. Table I shows the analyses, the pH of each untreated flour, and the chlorine treatment required to reach the final pH.

Photographs of cakes baked from the four varieties, shown in Fig. 1, illustrate the change of cake contour that occurs with increasing amounts of liquid. Three liquid levels at 6% increments have been found adequate for most samples. Some flours with wider tolerance to liquid changes may require baking at lower or higher liquid content if the full contour range from fallen to peaked is desired. Figure 2 shows the relation between cake volume and liquid level.

Flour from the Thorne and Clarkan varieties gave optimum volume and rounded contour at 103% liquid. Both gave fallen cakes at 97% liquid and peaked cakes with smaller volumes at liquid levels above 109%. The Clarkan series was extended to 121% to emphasize the critical nature of optimum liquid in some varieties. Other varieties such as Trumbull and Purkof show less abrupt volume change with

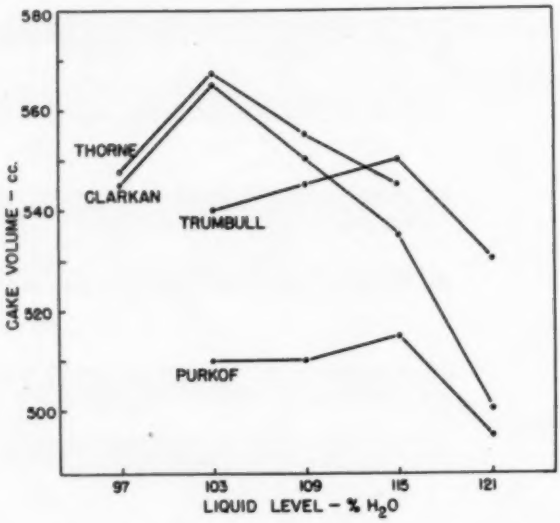

Fig. 2. Cake volume vs. liquid level plot, showing typical varietal differentiation with the lean formula.

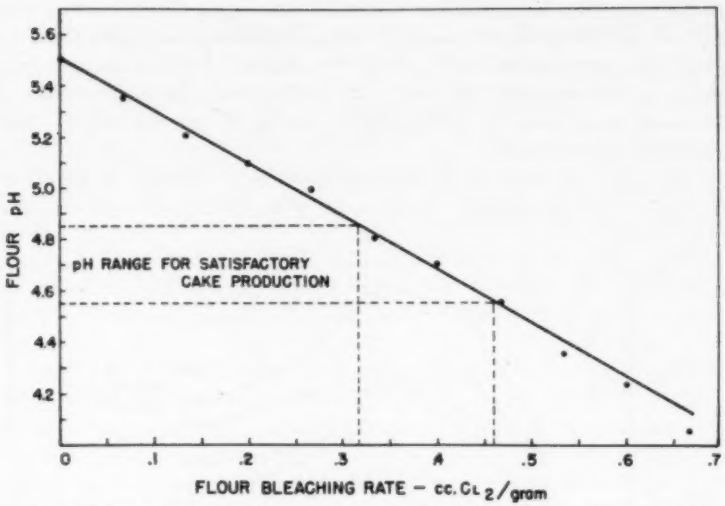

Fig. 3. Effect of chlorine treatment rate on final flour pH for a commercial short patent flour.

increasing liquid and illustrate the factor of relative liquid tolerance. Over-all characterization of varieties for cakemaking purposes is based on consideration of three objective factors: 1) volume of layers with rounded contour, 2) liquid level giving rounded contour, and 3) tolerance of volume and contour to changes in liquid content.

Effect of Bleaching. Early work with this method indicated a sensitivity to variable chlorine treatment as indicated by pH. To avoid erroneous results in varietal ranking studies, all flours must be bleached to give optimum performance. Figure 3 shows chlorine treatment rate (dosage) plotted against final pH for ten aliquots of an unbleached commercial short patent flour. The laboratory bleaching rate in cc/g may be converted directly to oz/cwt by multiplying the given value by a factor of 5.075.

A negative linear relationship occurred when individual 1500-g. samples were treated in a modified Wallace & Tiernan laboratory bleacher with increasing volumes of chlorine dioxide up to about 0.5 cc. per gram of flour (2.54 oz/cwt). Thereafter the pH change appears to accelerate, owing to greater bleaching efficiency of larger volumes of chlorine. Dotted lines in Fig. 3 indicate the pH range and corresponding chlorine treatment range that produced optimum cake-baking results.

Lean-formula baking results are summarized in Fig. 4. The lowest

curve represents layer volumes obtained with unbleached control flour of pH 5.5. Progressively heavier chlorine treatments resulted in increasing cake volumes until pH 4.8 was reached. Further increases did not result in larger cake volume, but at pH 4.7 and pH 4.6 there was an increase in tolerance to higher liquid levels. At pH 4.4 and 4.1 cake volumes were reduced.

Although the laboratory chlorine treatments yielding flours of pH 4.8–4.6 gave cake volumes approaching those obtained with the com-

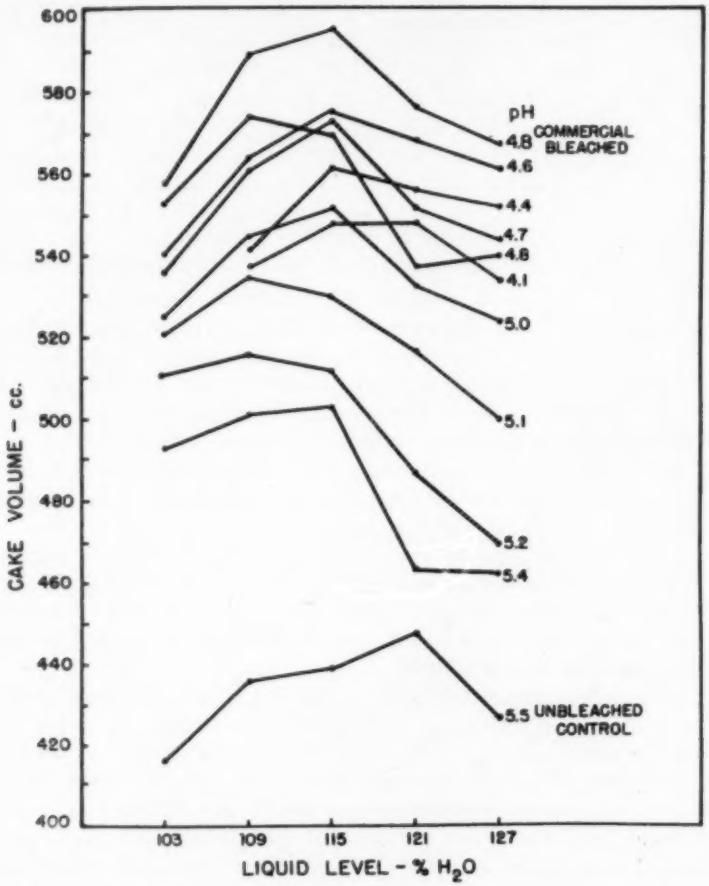

Fig. 4. Effect of chlorine treatment on cake volume. Commercial short patent flour using the lean formula method.

mercially milled and bleached control (pH 4.8), the latter were significantly larger at optimum liquid levels. Extent of bleaching did not appear to affect the optimum liquid requirement for this flour, since most pH levels gave maximum cake volume at 115% liquid.

Discussion

The lean-formula cake method has been used to classify selections and varieties of wheat for potential cake production. Extensive testing at this laboratory, and in cooperative work with several industrial groups, has indicated the method to be sensitive to quality differences and capable of correctly ranking various flours according to commercial test results. The lean formula has been used to evaluate soft wheat selections grown in nine eastern states in 1954, 1955, and 1956.

Sensitivity of the method to liquid tolerance and bleaching treatment has been presented. In addition, the method is sensitive to other experimental variables including particle size, length of patent extraction, and protein content differences. Replication error is small if day-to-day laboratory conditions are controlled, with particular attention to concentration and uniformity of the sugar solution.

Statistical analysis was performed on two sets of data. The standard deviation of cake volume estimated from duplicate bakes of 96 different samples was 3.8 cc. A second judgment of sampling errors was made from 104 bakes on a single flour at two liquid levels on 52 days. The analysis of variance indicated a standard deviation of 10.5 cc. with day-to-day variation included in the error. The removal of errors due to daily variations results in a standard deviation of 3.1 cc.

The lean formula, with a modified mixing method, is also being used in a fundamental fractionation and reconstitution study to determine the contribution and interactions of flour components on cake quality.

Acknowledgment

The writer wishes to acknowledge the assistance of D. C. Abbott and Miss Dorothy J. Barr, formerly of this laboratory, in the developmental stages, and of Ben D. Marshall in the baking studies reported.

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THE POLAROGRAPHIC DETERMINATION OF CYSTINE IN WHEAT AND FLOUR HYDROLYSATES[1]

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ABSTRACT

An improved method for the polarographic determination of cystine in wheat or flour hydrolysates has been developed. A calibration curve was established, based on the addition of increments of pure cystine to a hydrolysate in which the native cystine had been destroyed by treatment with hydrogen peroxide.

Optimum conditions for the hydrolysis of wheat and flour included autoclaving with 4*N* hydrochloric acid for 0.5 hour at 15 lb. pressure.

The average cystine content of flours milled from eleven wheats grown at eleven different locations was 1.88%, based on total protein content. This value is in close agreement with those determined by chemical and microbiological methods.

Specific methods of analysis for cystine involving both colorimetric and polarographic procedures have been the subject of numerous investigations. Brdicka (3) developed a polarographic method for the determination of cystine and adapted the procedure (4) to the determination of that compound in various proteins. A linear relationship was observed between wave height and concentration of cystine between $5 \times 10^{-6} M$ and $1 \times 10^{-4} M$. Kolthoff and Lingane (7) recommended $10^{-6} M$ to $2 \times 10^{-5} M$ concentrations of cystine.

Stern, Beach, and Macy (14), using the polarographic technique, prepared a new type of calibration curve by adding small amounts of cystine to a hydrolysate and estimating the cystine concentration from the wave heights obtained with and without the addition of pure cystine. Values were established for the cystine content of casein, edestin, and several globins which compared favorably with those obtained by colorimetric methods.

It has become apparent that the cystine content of proteins may be an important factor in biological systems (5,9,15). Since the analysis of this amino acid is difficult, it has been the object of this work to investigate the polarographic method of analysis with special attention to the preparation of an accurate calibration curve.

[1] Manuscript received March 28, 1958. Cooperative investigations between the Department of Chemistry, Kansas State College, and the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture. Published as contribution No. 448, Department of Chemistry, Kansas Agricultural Experiment Station, and No. 196, Department of Flour and Feed Milling Industries, Kansas Agricultural Experiment Station, Manhattan. A portion of a thesis presented in 1951 by Werner F. Daum as partial fulfillment of the degree Master of Science in Chemistry at Kansas State College.

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Development of Improved Polarographic Method

Materials. The polarographic analyses were performed with the use of a Sargent-Heyrovsky Model XII Polarograph, with a galvanometer sensitivity of 0.0054 microamps. per mm. A dropping mercury electrode with a drop time of 2.5 seconds was used.

All chemicals used in this investigation were of reagent grade. The sample of L-cystine used as a standard was 99+% pure. Its moisture content was 0.12%.

Polarographic Technique. Wöstmann's (15) method of measurement of the cystine wave was verified and used throughout this work. The measurement was based upon the difference between the diffusion current of cobalt and the minimum of the catalytic wave due to cystine. Below concentrations of $4 \times 10^{-5} M$ cystine, there was a linear relationship between wave height and concentration of cystine. A maximum suppressor such as caffeine was not used, since the cobalt diffusion current was reproducible.

The electrolytic medium containing $1.6 \times 10^{-3} M$ cobaltous chloride, $0.105M$ ammonium chloride, and $0.105M$ ammonium hydroxide was prepared in 500-ml. portions from stock solutions. Solutions suitable for assay were prepared by diluting 1.5 ml. of protein hydrolysate to 50 ml. with the electrolytic medium. The polarograms of these solutions were recorded within 5 minutes after thorough mixing.

Preparation of Hydrolysates. The method of Miller *et al.* (9) was employed in preparing a series of hydrolysates, utilizing different acid concentrations and different heating times. The hydrolysis time was recorded after a 15-lb. pressure had been reached in a preheated autoclave and ended at the beginning of the cooling process. Fifteen minutes' cooling time was allowed in all cases. The results are shown in Fig. 1. The polarograms of ground whole wheat hydrolysates prepared by autoclaving for less than 0.5 hour did not exhibit the characteristic maxima and minima of the catalytic waves and could not be resolved. Optimum conditions included $4.0N$ (13.7%) hydrochloric acid and an autoclaving time of 30 minutes. Further studies indicated that the same optimum conditions existed for the hydrolysis of flour. The shorter optimum hydrolysis time determined in this study, as compared with that reported previously (9), may be explained by the fact that the time recorded in the present investigation was based on the use of a preheated autoclave. These conditions are comparable with those of Singleton, Mertz, and Davis (11), who found that hydrolysis of alfalfa with $2.75N$ hydrochloric acid for 1 hour at 15 lb. pressure gave maximum cystine values, using microbiological assay methods. Sulfuric

acid was no better than hydrochloric acid as the hydrolyzing agent. It is likely that even the optimum hydrolysis curve (Fig. 1) represents some destruction of cystine. The optimum conditions probably indicate that the rate of cystine liberation from the protein has been overtaken by the rate of destruction of free cystine. In this connection it is noteworthy that Horn and Blum (6) and Balasubramanian and Ramachandran (1) found that a short hydrolysis time gave a truer measure of cystine content than a long hydrolysis period. The latter workers made a simultaneous study of the amino nitrogen and cystine released from rice and wheat and found that hydrolysis with 2.5*N* hydrochloric acid for 2.25–2.50 hours was the optimum period for hydrolysis.

All hydrolysates were prepared by the use of the optimum conditions established. One-gram samples of wheat or flour, on a moisture-free basis, were hydrolyzed with 25 ml. of 4*N* hydrochloric acid for 30 minutes at 15 lb. pressure in 125-ml. Erlenmeyer flasks covered with inverted beakers. After rapid cooling, the hydrolysates were made up to 100-ml. volumes and filtered. It was established that the acidity of

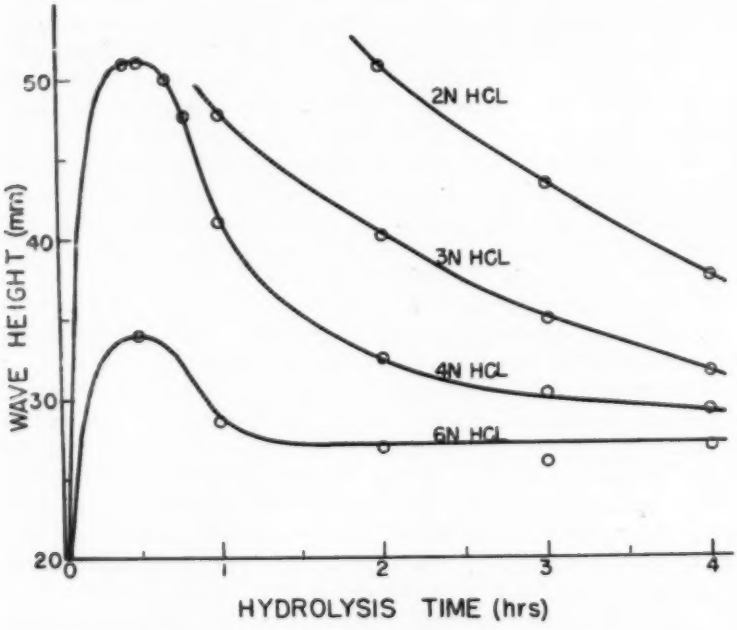

Fig. 1. Effect of hydrolysis time and acid strength on the rate of cystine liberation from wheat protein.

the resulting hydrolysate did not influence the cystine wave.

Preparation of a Standard Curve. Selective destruction of cystine and cysteine in a wheat or flour hydrolysate should allow the development of an absolute calibration curve, with the other constituents of the protein exerting their effect as in a normal hydrolysate. The method utilized by Lyman *et al.* (8) for destruction of cystine, cysteine, tyrosine, tryptophan, and methionine in protein hydrolysates for culture media was employed. A series of 25-ml. hydrolysates was treated with varying amounts of 1.0% hydrogen peroxide and allowed to stand at room temperature for 17 hours. Temperatures ranging from 18° to 40°C. caused no significant variation in the results. The hydrolysates were then heated 3 hours on a steam bath to destroy the excess hydrogen peroxide. This treatment resulted in complete elimination of the catalytic hydrogen wave of cystine. Polarograms recorded for cystine added to the hydrolysate produced a normal cystine wave. The effect of different concentrations of hydrogen peroxide on cystine wave height is shown below:

Hydrogen Peroxide, 1.0%	*Wave Height 10×10^{-4}M Cystine*
ml	*mm*
6	49.2
8	55.0
10	57.2
12	54.5
14	53.0

Two methods for the destruction of excess hydrogen peroxide were studied. The use of sodium sulfite gave conflicting results and was abandoned in favor of the heat treatment. The maximum cystine wave height was obtained when the hydrogen peroxide-treated hydrolysate was heated on the steam bath for 30 minutes. Further heating for 15 minutes resulted in identical results, but heating for 1 hour caused a slight decrease in the wave height. The data are shown below:

Heating Time	*Wave Height of 5×10^{-4}M Cystine*	*Wave Height of 10×10^{-4}M Cystine*
hours	*mm*	*mm*
0.25	33.0	51.6
0.50	34.3	56.0
0.75	33.8	55.1
1.00	32.5	53.5

Three wheat samples containing 10.5, 15.1, and 19.5% protein were hydrolyzed, filtered, treated with 10 ml. of 1% hydrogen peroxide, and

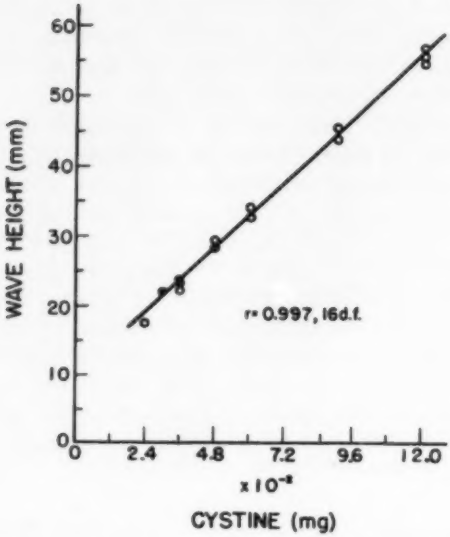

Fig. 2. A calibration curve for the polarographic determination of cystine in wheat and flour hydrolysates.

heated for 30 minutes. Polarograms were recorded for 1.5 ml. of each protein hydrolysate in the presence of various amounts of pure cystine. The calibration curve in Fig. 2 is a composite plot of the values obtained from the three hydrolysates. The equation of the line is: $Y = 4.65X + 9.77$, where Y is the wave height in mm. and X is the cystine concentration in mg. A slight curvilinear relationship was observed at cystine concentrations greater than those shown.

Recovery Experiments. Recovery experiments were performed by preparing hydrolysates of flour with and without the addition of pure cystine before hydrolysis. A polarogram was obtained directly on the hydrolysate to obtain a measure of the *in situ* cystine. A polarogram also was obtained on the hydrolysate prepared with added cystine to determine total cystine. The recovery percentage was based on the difference between these two polarographic wave heights, referring to the calibration curve previously described (Fig. 2). The average recovery of cystine was 99.1% for nine determinations, using the average of four individual polarograms for each determination.

Factors Affecting Wave Height. Several components of biological material may influence the catalytic wave of cystine. Wöstmann (15) reported that starch caused considerable destruction of cystine during

hydrolysis. To determine the loss under the hydrolysis conditions established for this work, 8-mg. samples of cystine were hydrolyzed with amounts of starch ranging from 0.0 to 1.0 g. The wave heights recorded for these samples were identical; hence, there would appear to be no destruction of cystine during hydrolysis for 0.5 hour at 15 lb. pressure. The long hydrolysis time in the presence of strong hydrochloric acid may account for the destruction of cystine reported by other workers.

The presence of other amino acids may also complicate the analysis of cystine. Since tyrosine, tryptophan, and methionine are destroyed by treatment with hydrogen peroxide, the influence of these amino acids on the cystine wave was studied. Methionine had no effect. This result with methionine corroborated the work of Smith and Rodden (13) and Sládek and Lipschütz (12). Tryptophan also did not suppress the wave due to cystine in the presence of hydrogen peroxide-treated hydrolysates in concentrations up to four times that present in wheat. Tyrosine was found to suppress the catalytic wave only at three times its normal concentration in wheat. Amino acids other than those destroyed by hydrogen peroxide and which are normally present in wheat also were found to have no significant additional effect on the cystine wave. This was shown by the similarity of standard curves prepared by using hydrogen peroxide-treated hydrolysates representing protein levels ranging from 10 to 20%. It would appear, therefore, that the presence of varying quantities of starch and the various component amino acids of wheat need not be considered in the analysis of cystine in wheat or flour under the experimental conditions employed.

Application of Method to Determination of Cystine in the Protein of Hard Red Winter Wheat Flour

The cystine content of the protein of two sets of flour samples was determined in duplicate by the established method of analysis. One set consisted of eleven varieties composited by location from eleven different locations. The other set consisted of the same eleven varieties composited by variety from the same eleven different locations. A summary of the assay data and the analyses of variance are shown in Table I. Differences in cystine content in both variety and location composites were significant at the 1 and 5% levels, respectively. The least significant mean differences between means of duplicate determinations were 0.13 and 0.12 for variety and station composites, respectively. In the variety composites, the percent cystine in the protein ranged from 1.76 to 2.02% and averaged 1.88%. In the location composites, the percent cystine in the protein ranged from 1.68 to

2.05% and averaged 1.86%. Since each of the two series contained flours composited in a different manner, the good agreement of the data lends considerable confidence to the recommended method. The standard deviation for the data was 0.06% cystine, and the coefficient of variation was 3.2%. Additional substantiation of the procedure is found in data reported for the cystine content of flour protein by other workers. The cystine content of flour protein obtained by chemical

TABLE I

SUMMARY OF POLAROGRAPHIC ASSAY DATA AND ANALYSES OF VARIANCE OF THE PERCENT CYSTINE IN THE TOTAL PROTEIN OF 22 FLOUR SAMPLES COMPOSITED BY VARIETY AND LOCATION

Variety	Protein [a]	Cystine [b]	Location	Protein [a]	Cystine [b]
	%			%	
Pawnee	15.3	2.02	Manhattan	14.3	2.05
Comanche	15.6	1.96	Hutchinson	14.6	2.04
Blue Jacket	16.1	1.93	Kingman	16.5	1.90
Red Chief	14.9	1.91	Tribune	14.5	1.89
Turkey	14.1	1.88	Garden City	15.8	1.88
Kiowa	15.7	1.87	Wichita	16.7	1.86
Wichita	14.4	1.87	Belleville	15.1	1.86
Westar	14.3	1.85	Colby	15.7	1.85
Ponca	14.7	1.81	Dodge City	17.7	1.73
Triumph	15.6	1.78	Mankato	12.6	1.72
Blackhull Sel.	15.0	1.76	Liberal	14.0	1.68
L.S.D.		0.13	L.S.D.		0.12

ANALYSES OF VARIANCE

Source of Variation	Degrees of Freedom	Mean Square	Source of Variation	Degrees of Freedom	Mean Square
Variety	10	0.0114*	Location	10	0.0288**
Error	11	0.0037	Error	11	0.0032

[a] Moisture-free basis.
[b] Mean for two separate analyses.

methods has been reported as $1.9 \pm 0.1\%$ (2). Pence *et al.* (10), employing a colorimetric method, also reported an average of $1.9 \pm 0.1\%$ cystine for the gluten washed from a series of 17 different flours.

Wöstmann (15) obtained percentages of cystine in flour protein ranging from 2.2 to 4.5%, employing a technique similar to that used by Stern, Beach, and Macy (14). This variation in reported values may be associated, in part, with the variation in cystine among wheat samples. Significantly different results may also be associated with the method employed, since the average cystine content of flour protein was 2.20% for the samples analyzed in the present study when a calibration curve was prepared according to the method of Stern, Beach, and Macy (14).

Improved Procedure for the Polarographic Determination of Cystine in Biological Materials

The improved procedure which was developed as a result of these studies is presented below:

Preparation of Hydrolysates. Heat 1.0 g. of wheat or flour with 25 ml. of 4.0*N* hydrochloric acid for 0.5 hour at 15 lb. pressure in a preheated autoclave. Cool rapidly, dilute to 200 ml., and filter.

Apparatus. Recording Polarograph.

Reagents. 1. Cobaltous chloride, $8.0 \times 10^{-3} M$. Store in refrigerator.

2. Ammonium chloride, 1.05*N*. Store in refrigerator.

3. Ammonium hydroxide, 1.05*N*. Store in refrigerator.

4. The electrolytic medium containing $1.6 \times 10^{-3} M$ cobaltous chloride, 0.105*M* ammonium chloride, and 0.105*M* ammonium hydroxide was prepared fresh each day in 500-ml. portions from stock solutions.

5. Cystine stock solution: Dilute 240.3 mg. of L-cystine to 1000 ml. with water. Two milliliters of stock solution diluted to 50 ml. yield a $4 \times 10^{-5} M$ cystine solution.

6. Clean mercury.

Procedure. Dilute 3 ml. of the filtered hydrolysate to 50 ml. with the electrolytic medium. Record polarograms within 5 minutes after thorough mixing. Measure the cystine wave height as the difference in mm. between the diffusion current of cobaltous ion, obtained from electrolyte with no added cystine, and the minimum of the catalytic wave due to cystine. (See Wöstmann, B. Cereal Chem. *27*:391; 1950.)

Preparation of Standard Curve. Add 10 ml. of 1% hydrogen peroxide to the filtered hydrolysate. After the solution has stood for 17 hours at a temperature between 18° and 40°C., heat it on a steam bath for 30 minutes to destroy excess hydrogen peroxide. Record polarograms for 3.0-ml. aliquots of the hydrogen peroxide-treated protein hydrolysate with amounts of added cystine, ranging from 2.0 to 12.0×10^{-2} mg. in a total volume of 50 ml. of electrolytic medium. Plot wave height against added cystine.

NOTES.

1. Use of too much hydrolysate will eliminate the wave minimum.
2. Mercury should not be permitted to be in contact with electrolytic solution for more than 5 minutes prior to running the polarogram.
3. The normality of ammonium hydroxide should be rigidly controlled.

Discussion

Certain difficulties not mentioned by other workers were experienced with the dropping mercury capillary of the polarograph. The maximum of the catalytic wave exhibited large irregular fluctuations which made certain polarograms difficult to measure. This was true particularly for those samples with high protein content. Periodic cleaning of the capillary with concentrated hydrochloric acid virtually eliminated this effect and indicated that such irregularities were caused by adherence of some components of the hydrolysate to the capillary.

A measurable polarogram was recorded only when the proper amount of hydrolysate was employed. Hydrolysates containing little cystine did not exhibit a minimum when too much hydrolysate was used. This effect may be due to the suppressing action of the amino acids on the catalytic wave of cystine. This difficulty can be corrected either by decreasing the sensitivity of the galvanometer, which causes a reduction of the resulting wave height, or by decreasing the amount of hydrolysate.

The standard deviation for ten replicate determinations of cystine performed in sequence with a freshly prepared hydrolysate was 1.0 mm. difference in wave height. This value corresponds to a standard error of 4%. Similar determinations, made on the same hydrolysate on ten successive days, resulted in a standard deviation of 1.3 mm. and was equivalent to 6% standard error.

The method described was applied only to the measurement of cystine in wheat and flour; however, it appears reasonable to assume that it could be adapted to the measurement of cystine in other biological materials. The optimum conditions for establishing the calibration curve, particularly the hydrolysis step and the peroxide destruction step, may be different for other materials.

Acknowledgment

The L-cystine used in this investigation was furnished by Merck & Co., Inc., Rahway, New Jersey.

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GRAIN STORAGE STUDIES
XXVII. The Inorganic Phosphorus Content of Deteriorating Wheat[1]

R. L. GLASS AND W. F. GEDDES

ABSTRACT

Deteriorating wheat exhibited an increase in inorganic phosphorus content, the rate of which was greater with increasing temperatures and moisture contents. Accompanying this change was a decrease in phytic acid phosphorus. The inorganic phosphorus content of a sample of wheat increased from an initial value of 0.021% to 0.067% and 0.059% when stored at approximately 18% moisture under nitrogen and air respectively for 26 weeks at 30°C.

The observed changes are apparently due to the action of wheat phytase upon phytic acid. Increased levels of inorganic phosphorus might serve as a useful index of previous poor storage conditions, such as a brief exposure to high moisture and temperature, with a resulting poor future storage behavior.

During an investigation of biochemical changes which occur in wheat stored at high moisture levels in an atmosphere of nitrogen in this laboratory, it was observed that inositol, found in barely detectable quantities in sound wheat, was present in readily isolatable quantities in spoiled wheat. Since wheat contains the enzyme phytase (8), which catalyzes the hydrolysis of phytic acid (inositol hexaphosphoric acid) to inositol and inorganic phosphate, it appeared likely that the inositol formed during wheat deterioration was the result of phytase activity and that considerable amounts of inorganic phosphorus must be liberated. Accordingly, inorganic phosphorus analyses were performed on wheat samples stored in air and nitrogen and which were in various stages of deterioration. The results are reported here.

Materials and Methods

The wheat samples used were available as a result of several storage experiments which had been conducted to determine the effect of nitrogen, moisture content, temperature, and various molds on the keeping quality of hard red spring wheats. They will be described in presenting the results.

Inorganic phosphorus was determined by the method of Pons and Guthrie (6). A sample (1.0 g.) of the ground material (micro-Wiley mill, No. 60 screen) was extracted for 1 hour at room temperature with 50 ml. of 0.75*N* trichloroacetic acid solution. An ammonium molybdate reagent was added to a suitable aliquot; this was followed

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by reduction with stannous chloride and colorimetric determination of the resulting blue color at 730 mμ. All figures are averages of duplicate determinations and are expressed on a dry-weight basis.

Phytic acid phosphorus was determined by the method described by McCance and Widdowson (4). A 5-g. sample of wheat, ground as for the inorganic phosphorus assay, was extracted with 0.5*N* hydrochloric acid solution for 2 hours. Phytic acid was precipitated from the partially neutralized extract by the addition of ferric chloride. The washed precipitate was dissolved in alkali and hydrolyzed by boiling in a sulfuric acid-perchloric acid solution, and the inorganic phosphorus determined as outlined above.

Free fat acidity was determined on a diethyl ether extract (6 hours) of 3 g. of ground meal (micro-Wiley mill, No. 40 sieve) according to the method of Ames and Licata (1) as modified by Hunter *et al.* (3). The results are expressed as mg. potassium hydroxide per 100 g. dry wheat.

Viability was determined by the Minnesota State Seed Testing Laboratory. Two hundred seeds were placed on a wet blotting paper supported by a wire tray in a germinator maintained at 20°C. The seeds which had normal sprouts after 7 days were considered viable.

Results

A summary of some of the changes which occurred in a sample of Montana Marquis wheat stored in air for 24 weeks at 18% moisture and 20°C. is given in Table I. As viability decreased, inorganic phosphorus and free fat acidity steadily increased to final values approximately twofold and fourfold, respectively, over the initial values. This parcel of wheat was heavily infested with storage molds by the fourth week.

Table II gives the results obtained during storage trials of samples of Ceres wheat held at 30°C. and at approximately 18% moisture (2).

TABLE I

CHANGES IN WHEAT STORED IN AIR AT 18% MOISTURE AND 20°C.

STORAGE TIME	VIABLE SEEDS	INORGANIC PHOSPHORUS	FREE FAT ACIDITY [a]
weeks	%	%	
0	98	0.021	20.0
4	88	0.024	39.5
8	56	0.026	45.1
12	18	0.030	62.5
16	13	0.032	68.4
20	10	0.037	82.7
24	7	0.041	82.8

[a] Milligrams of KOH required to neutralize the free fatty acids in 100 g. wheat, dry-weight basis.

Prior to storage the wheat had been surface-sterilized by immersion for 30 seconds in a 0.5% aqueous solution of sodium hypochlorite, rinsed briefly in sterile distilled water, and air-dried overnight at room temperature. It was then divided into several sublots, each of which was adjusted to 18% moisture by the addition of sterile water, or with water containing spores of either *Aspergillus restrictus* or *A. flavus*.

TABLE II

CHANGES IN WHEAT STORED WITH VARIOUS MICROORGANISMS AT 18% MOISTURE[a] AND 30°C.

Storage Time	Viable Seeds			Inorganic Phosphorus			Free Fat Acidity		
	Mixed[b]	*A. restrictus*[b]	*A. flavus*[b]	Mixed	*A. restrictus*	*A. flavus*	Mixed	*A. restrictus*	*A. flavus*
weeks	%	%	%	%	%	%			
0	97	97	97	0.021	0.021	0.021	13.0	13.0	13.0
1	94	94	94	0.031	0.025	0.040	13.0	18.0	17.3
2	82	31	23	0.031	0.032	0.043	32.0	33.1	52.4
5	41	10	10	0.031	0.036	0.048	60.1	88.9	81.6
13	14	9	1	0.041	0.046	0.057	62.7	73.5	102.3

[a] Average weekly moisture contents were 16.9%, 17.3%, and 18.9%, respectively, for the mixed, *A. restrictus*, and *A. flavus* samples.

[b] The samples were surface-sterilized and inoculated with either sterile distilled water, *A. restrictus*, or *A. flavus*. A mixed group of storage molds developed in the sample inoculated with sterile distilled water.

In all cases there was a rapid decline in viability accompanied by an increase in inorganic phosphorus and free fat acidity. The changes were more rapid than those observed at 20°C., and again, free fat acidity increased more during the observation period than did inorganic phosphorus. It is notable, however, that changes in the latter were observed by the end of the first week of storage and prior to changes in either viability or free fat acidity. The wheat inoculated with *Aspergillus flavus* appeared to undergo more rapid increases in free fat acidity and inorganic phosphorus than did the other samples, but that was undoubtedly due to the sample's having an average moisture content of 18.9% during the experiment, as opposed to 16.9% and 17.3% for the other two samples. Similar data for wheat stored at 16°C. revealed that the inorganic phosphorus increased at a rate of about 40% of that observed at 18% moisture.

That the observed changes were, indeed, a result of phytic acid hydrolysis was determined by assaying a sample of wheat which had been stored for 24 weeks at 18% moisture and 30°C. for phytic acid phosphorus and inorganic phosphorus. A sample of the original wheat, which had been held at −10°C. during the storage period, was similar-

ly assayed. The data given below strongly indicate that the increase in inorganic phosphorus resulted from the hydrolysis of phytic acid.

	Sound Wheat	*Spoiled Wheat*
	%	%
Phytic acid phosphorus	0.210	0.150
Inorganic phosphorus	0.021	0.065
Total	0.231	0.215

The samples of wheat used thus far had been heavily contaminated with molds, and the changes observed might well have occurred as a result of mold activity. There was available a parcel of wheat which had deteriorated during storage in an atmosphere of nitrogen at 30°C. and 18% moisture. The sample had remained mold-free during the 26-week storage period in contrast to a control sample stored under similar conditions in an atmosphere of air. These two samples together with a sample of the original wheat were assayed for inorganic phosphorus and free fat acidity as before.

As shown in Table III, the mold-free sample also exhibited an increase in inorganic phosphorus which was, in fact, somewhat greater than that of the mold-infested sample. The changes in fat acidity exceeded those in inorganic phosphorus, particularly in the mold-infested sample.

Two samples of spoiled wheat obtained from commercial bins were each separated, on the basis of germ discoloration, into a sound and a

TABLE III

CHANGES OCCURRING IN MOLD-INFESTED AND MOLD-FREE WHEAT

	VIABLE SEEDS	FREE FAT ACIDITY	INORGANIC PHOSPHORUS
	%		%
Original	97	17	0.021
Mold-infested[a]	0	127	0.059
Mold-free[b]	0	71	0.067

[a] Stored in an atmosphere of air at 30°C. and 18% moisture.
[b] Stored in an atmosphere of nitrogen at 30°C. and 18% moisture.

TABLE IV

INORGANIC PHOSPHORUS CONTENT OF "SICK" AND SOUND WHEAT

SAMPLE	ORIGINAL	INORGANIC PHOSPHORUS	
		"Sick"[a]	Sound[a]
	%	%	%
1	0.062	0.071	0.036
2	0.059	0.065	0.042

[a] The original sample was separated into a "sick" and sound portion on the basis of germ discoloration.

"sick" fraction. These were then each assayed for inorganic phosphorus as before. The results, given in Table IV, indicate that those kernels which have undergone the most extensive deterioration have suffered a greater amount of phytic acid hydrolysis, possibly because at some time during storage localized damp spots had occurred with a resulting increase in phytase activity.

Discussion

These results indicate that, in wheat stored under conditions of elevated moisture and temperature, changes occur which cannot be ascribed to mold activity. Although free fat acidity consistently rose during storage to a greater extent than did inorganic phosphorus, the changes in the latter appeared to be more rapid in the very early stages of rapidly deteriorating wheat. Wheat phytase has been shown (5) to have a temperature optimum of 55°C., and it would be expected that more marked changes in the inorganic phosphorus content of stored wheat would be observed at temperatures above those of 20°C. and 30°C. used here and which resulted in noticeable changes as early as 1 week from the beginning of storage.

Sorger-Domenigg *et al.* (7) observed that when wheat was exposed to a moisture content of 21% for 1 week's time, its storage life, after drying to 14% moisture, would be markedly shortened. Such a brief exposure to high moisture would probably not affect the free fatty acid content. However, the results given here indicate that the level of inorganic phosphorus might be affected, and the ratio of inorganic phosphorus to total phosphorus might prove to be a useful index of previous poor storage conditions and future poor storage behavior.

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A SIMPLE METHOD FOR DETERMINING THE PROTEIN CONTENT OF WHEAT AND FLOUR SAMPLES[1]

LOUIS FEINSTEIN[2] AND JOE R. HART[2]

Many laboratories desire to determine the protein content of wheat and flour samples by a method simpler than the Kjeldahl method and which has at the same time a fair degree of accuracy. Three such methods having great promise have been described recently. Pinckney (4) successfully applied the biuret reaction to the quantitative estimation of protein in wheat and wheat flour. Udy (5,6) has estimated the protein in wheat and flour by ion-binding, using the dye-binding capacities of wheat proteins. Zeleny (7,8) used a phosphate buffer in his photometric method to develop a colloidal suspension from the protein peptized with alkali. In a review of the literature it was noted that sulfosalicylic acid had been used in the biological field as a reagent to determine the protein content of such fluids as blood and serum (1,2,3). In experiments with this reagent for determining the protein content of wheat and flour samples, a simple method was developed which gives reproducible results. No reagents are used which are of a dangerous concentration, and the method is quick and requires no heating or distilling.

Materials and Procedures

Briefly, the sample (ground in the case of wheat) is extracted with dilute alkali. After the extract has been centrifuged, an aliquot of the supernatant fluid is reacted with sulfosalicylic acid solution to produce a slightly opalescent liquid. The optical density of this liquid is then determined in a photoelectric colorimeter. The optical density readings are translated into percent protein by means of a calibration curve.

Samples. The wheat samples included U. S. wheats of the following types: Western white, Eastern white, soft red winter, hard red winter, and hard red spring. Commercial flour samples routinely tested by the Division were used and included soft, all-purpose, and hard flours. The laboratory of the Grain Division made the Kjeldahl protein determinations ($N \times 5.7$) for all samples.

Method. One gram of flour or ground wheat[3], weighed with an

[1] Manuscript received April 28, 1958.
[2] Biological Sciences Branch, AMS, U. S. Dept. of Agriculture, Beltsville, Md.
[3] The wheat samples should be finely ground with the grind of uniform granulation.

accuracy of ±5 mg., was placed in a flask, 100 ml. of 0.05*N* sodium hydroxide were added, and the flask was shaken mechanically for 15 minutes. Suspended material was separated from the solution by centrifuging at a speed approximately 6,000 r.p.m. for 10 minutes or until a clear supernatant solution was obtained. By means of a pipet, 5 ml. of the solution were transferred to a glass-stoppered graduate cylinder and sulfosalicylic acid (30 g. per liter) was added to fill to the 50-ml. mark. The cylinder was then inverted two or three times to ensure uniform distribution of the colloidal suspension formed in the solution.

A Klett-Summerson[4] photoelectric colorimeter, in conjunction with a 540 $m\mu$ filter, was used for measuring the degree of light transmittance and, indirectly, the degree of turbidity of the solution. The 20 by 40-mm. cell was so placed that the beam of light traversed the 40-mm. dimension. Some of the sulfosalicylic acid served as the blank for zero adjustment of the instrument.

With samples of high protein content, the colloid which forms when the acid is added appears to undergo a change in state of aggregation upon standing. Therefore, the colorimeter reading should be made immediately after the sulfosalicyclic acid solution is added.

Results

Figure 1 is a plot of Klett-Summerson colorimeter readings as a function of protein content for 103 samples of commercial flours; the same relationship is shown for 99 samples of wheat. The regression equation for the flour samples is:

$$y = 2.53 + 18.20X$$

and for the wheat samples:

$$y = -2.99 + 18.02X$$

where y is the colorimeter reading and X is the percent of protein. The regression line is drawn in each plot. The standard deviation from the regression line in terms of percent of protein is 0.25 for flour and 0.29 for wheat. These equations may be used to construct tables by means of which colorimeter readings may be translated into percent of protein.

An examination of the regression lines reveals that when it is extrapolated to 0% protein, the colorimeter reading will be very close to zero, thus indicating that colorimeter readings are very little affected by any factor except protein content.

[4] The mention of specific instruments or trade names is made for the purpose of identification and does not imply any endorsement by the United States Government.

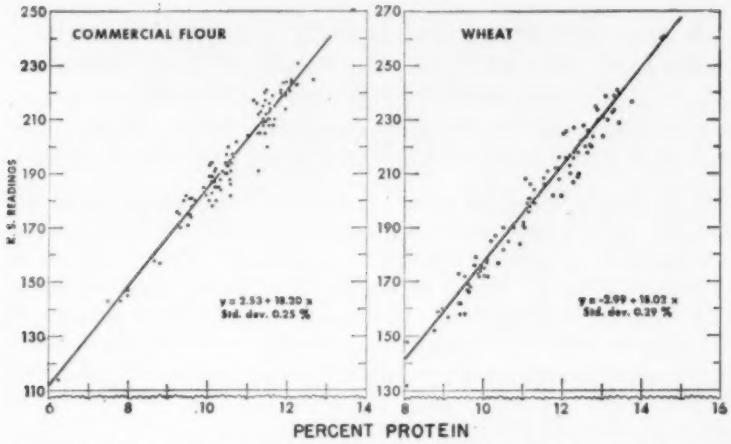

Fig. 1. A plot of Klett-Summerson readings as a function of percent of protein for 103 samples of commercial flour and for 99 samples of wheat.

Acknowledgment

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A NOTE ON THE RADIOACTIVITY DISTRIBUTION IN MILLING PRODUCTS OF THATCHER WHEAT GROWN IN THE PRESENCE OF STRONTIUM-90[1]

C. C. LEE[2]

The contamination of plant products by strontium-90 from fall-outs of nuclear explosions is of considerable current interest. In conjunction with a study on the uptake of Sr-90 by wheat plants of the Thatcher variety, the results of which will be reported elsewhere, we have studied the radioactivity distribution in the milling products of two crops of kernels remaining from two series of experiments. These kernels were derived from a number of plants grown individually in the greenhouse. At various stages of growth, 1 or 2 ml. of a solution of strontium chloride (0.1 mg. per ml.) containing Sr-90 were introduced into the soil. The kernels were harvested at maturity. The millings of the two crops, each weighing about 7 g., were carried out in a mill originally designed for 100-g. wheat samples as described by Geddes and Frisell (1). The radioactivity and ash contents of the flour, shorts, and bran from each crop were analyzed. For the radioactivity assays, portions of various samples, each containing 5.0 mg. of added inactive strontium chloride as carrier, were first wet-ashed in concentrated sulfuric acid and perchloric acid. After all organic matters were destroyed and practically all the excess mineral acids evaporated off, each residue was taken up in water, neutralized, and made slightly basic before the strontium was precipitated as strontium phosphate. The precipitate was collected by centrifugation, transferred to a sample pan, and dried;

TABLE I

STRONTIUM-90 ACTIVITY AND ASH CONTENTS IN MILLING PRODUCTS

FRACTION	WEIGHT	MOISTURE	DRY MATTER	ASH (DRY BASIS)	TOTAL ASH IN FRACTION	SR-90 ACTIVITY[a]
	g	%	*g*	%	*mg*	%
Crop 1						
Flour	3.4	11.3	3.0	0.70	21	9
Shorts	1.3	10.1	1.2	3.07	37	29
Bran	2.3	30.6	1.6	5.11	82	62
Crop 2						
Flour	3.6	11.4	3.2	0.93	30	16
Shorts	1.7	9.1	1.5	2.72	41	31
Bran	1.4	11.8	1.2	4.70	65	53

[a] Total activity in all fractions = 100%.

[1] Manuscript received September 29, 1958.
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and the activity was counted. The results are recorded in Table I. For both crops, the bran contained the largest and the flour the least amounts of radioactivity. This is not surprising, as the bran has the highest mineral matter as measured by its ash content. For all the fractions analyzed, higher ash contents are associated with greater amounts of Sr-90 activity as shown by Fig. 1. These results appear to indicate

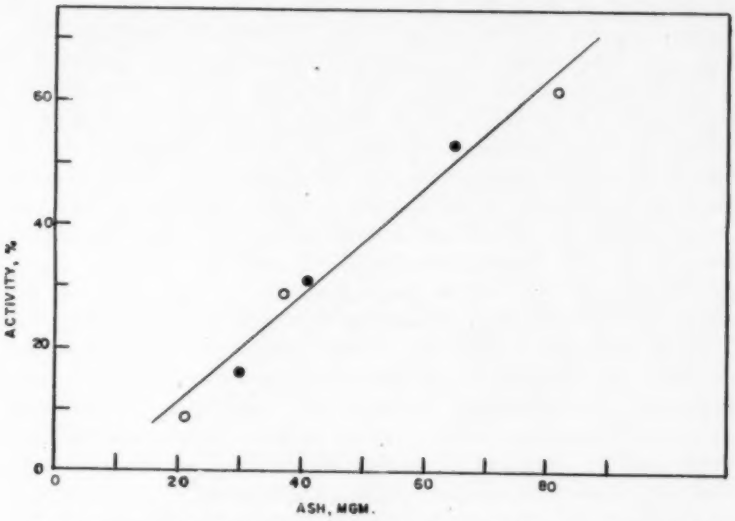

Fig. 1. Percent of Sr-90 activity in various fractions of milled products vs. weight of ash in corresponding fractions. Open circles, crop 1; solid circles, crop 2.

that one may reasonably expect lower degrees of contamination by Sr-90 from uptake of fall-out debris for higher-grade flours with lower ash contents.

This work was generously supported by the Saskatchewan Research Council and the Saskatchewan Agricultural Research Foundation.

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A NOTE ON METHODS OF DISPERSING FIFTY-PERCENT-AMYLOSE CORN STARCH[1]

J. W. Sloan, Majel M. MacMasters, and F. R. Senti

Corn starch with an amylose content of 50% (1,8) is becoming available (5) in increasing quantity, and many of its projected uses require dispersion with minimum loss of desirable properties. Amylose and amylopectin, fractionated (4) from ordinary corn starch, are readily dispersed homogeneously in water by first saturating the water with a dispersant, such as butanol or Pentasol,[2] and then refluxing the mixture for 10 minutes. In strong contrast to the behavior of amylose, amylopectin, and the ordinary corn starch granule, the high-amylose corn starch granule is highly resistant to the action of aqueous media (2) with or without dispersants. Even at temperatures of 70°–80° higher than the 92°C. used for complete dispersion of amylose, high-amylose starch granules are undispersed. At still higher temperatures, degradation and browning become severe.

Laboratory studies have shown that a mild alkali prewash renders the corn starch granule containing 50% amylose amenable to aqueous dispersion at temperatures between 140° and 170°C. High-amylose corn starch (wet-milled from a sample of Bear Amicorn (5)), slurried in ten times its weight of 5% sodium bicarbonate (or carbonate) solution and stirred 5–20 minutes at 25°C., was recovered by filtration and washed once by slurrying in distilled water and refiltering. A 2% slurry of this product (namely, alkali-prewashed starch) in distilled water had a pH of 9.4, which dropped to 8.0 on subsequent autoclaving.

Aqueous dispersions of pretreated high-amylose corn starch have been made with and without dispersants. In the first case, 27 ml. of *n*-butanol (or Pentasol) and 180 ml. of distilled water were mixed with 20 g. of starch and heated in a suitable shaker-equipped pressure vessel to a temperature of 145°–150°C., which was maintained for approximately 10 minutes. The vessel was then cooled to 90°, opened, and its contents transferred to a preheated distilling flask, where the dispersant was removed by azeotropic distillation. In the absence of dispersant, a temperature of 160°–165°C. for 1–5 min. was necessary.

[1] Manuscript received April 7, 1958. Contribution of the Northern Utilization Research and Development Division, Peoria, Illinois, one of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture.

[2] Mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

This high-amylose starch without pretreatment was also rapidly dissolved in formamide (4) (or monoethanolamine) by heating a 10–15% mixture to 150°C. The solubilized product was recovered by ethanol precipitation and could then be dispersed by the butanol-water reflux method.

The dispersions were tested and compared as follows: Transparent self-supporting films were made after the method used by Wolff and co-workers (6). The hot dispersions were filtered, spread on silicone-coated glass plates, and allowed to dry to an average thickness of 0.030 mm. After equilibration in a constant-humidity room, each film was tested for tensile strength, ultimate elongation, folding endurance, and burst and tear factors in accordance with ASTM procedures. The results are compared in Table I with those for film made from amylose and from a 1:1 mixture of fractionated amylose and amylopectin previously studied by I. A. Wolff *et al.* (6).

TABLE I
FILM PROPERTIES

PREPARATION	TENSILE STRENGTH	ELONGATION	DOUBLE FOLDS	BURST	TEAR	INTRINSIC VISCOSITY
	kg/sq mm	%		*points*	*g*	
Amylose, BuOH-HOH reflux	7.0	14.0	853	21	7.3	...
Amylose:amylopectin, 1:1	6.9	8.7	108	14	5.5	...
Starch, 50% amylose						1.18
Prewash, BuOH-HOH, 146°	6.5	5.4	77–107	14	6.6	1.05
Prewash, water, 160°	6.3	6.0	79	16	...	0.91
Formamide, 150°	6.5	10.4	107	19	6.9	0.92

Microscopic examination of the starch films showed the absence of granular shapes which often appear in films from poorly dispersed starch. Also the formamide-prepared film had no surface roughness, was perfectly clear, did not gel, and could be cast cold; whereas the other films had varying degrees of haziness and gelled before drying. The films swelled in water and lost most of their strength, but remained intact except the formamide preparation, which was soluble.

An estimate of the extent of degradation which resulted from the dispersion conditions was obtained by determining intrinsic viscosity of the films and of the 50% amylose starch. Samples were dissolved in 1*M* potassium hydroxide at 7°C., and relative viscosity measurements were made with the Ostwald-Cannon-Fenske Viscometer at 25.10° ± 0.03°C. The plot of inherent viscosity (ratio of natural logarithm of the relative viscosity to concentration) against concentration of sample solutions, when extrapolated to zero concentration, gave intrinsic viscosities (7) shown in Table I.

Acknowledgment

Valuable assistance in the testing of films and measuring of viscosity was rendered by Clark Himmel.

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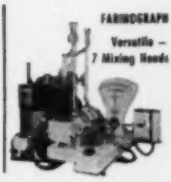

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